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Microscopy of Synapses of Non-Excised Frog Heart. (19636)

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The synapses of beating but excised frog heart have been studied by Fedorov(1), with the Gramenitsky preparation(2,3). This was made possible by stretching of the atria and sinus venosus, and dissecting away of adventitious tissue. These portions of the heart are then sufficiently translucent for microscopic study of the detailed structure of the synapses with the ganglion cells, which lie in these portions of the heart. It is also feasible, however, to subject the synapses to microscopy without removing the heart from the body or severing the vagi. A note limited to the procedure and apparatus required and the range of its applications will appear elsewhere. It may be noted here that they serve to stretch and transilluminate these portions of the heart with the organ *in situ*. Under these conditions, the terminations of the vagus and the ganglion cells on which they synapse are more viable, and display the following characteristics.

The ganglion cells. In a fresh preparation of the heart *in situ,* untreated except with Ringer's, and viewed with an oil-immersion objective and Ringer's in place of oil, the ganglion cells are concealed in opaque white capsules of connective tissue. The preganglionic terminals and end-feet are outlined as fine linear and vesicular elevations of the surface. After many hours, often up to 6 or longer, the opacity may diminish by itself, and the cell itself gradually becomes visible in detail. The cytoplasm then reveals a distinct granulation, but no cell membrane is ever to be seen. The nucleus presents itself centrally located, with its membrane and nucleolus both sharply defined. Eventually the nucleus moves to a side, very frequently in an *axofugal* direction. The axon frequently is bent on itself to approximate the border of the cell for a short distance. The connective tissue capsule remains visible in part, as a bracelet which closely encircles the cell. One or more fibroblasts are to be seen in the capsule, in addition to one frequently near the flexure of the axon.

On the other hand, in the *excised* preparation, the opacity of the capsule has often disappeared by the time of mounting, and if not, the translucency and enhanced cell visibility develop in a shorter period than in the non-excised heart. It is to be concluded from the time required to mount the excised heart, that morphologic deterioration of the cells begins within an hour of severance of the preganglionic trunks and abolition of the circulation.

The Vagal Terminals and End-Feet. With emergence of cellular detail in the heart *in situ*, the presynaptic structures also become visible. The terminals of the vagus are extremely fine and can be discerned for short distances only. The end-feet are tiny spheroids and occur in clusters on the cell. Both structures retain their morphology for hours. Eventually many of the fibers and end-feet become invisible or disintegrate, while other end-feet become slightly enlarged and on some cells grossly swollen. In the *excised* heart, on the other hand, the visible fibers and end-feet are fewer by the time that the mounting is accomplished, and a slight enlargement of most end-feet has already taken place. It is to be concluded that vagal terminals and end-feet, like the ganglion cells, begin to deteriorate within an hour of sectioning of the trunk and abolition of the circulation.

Enhancement of Visibility by Acidification. The enhanced visibility of cellular and presynaptic structures which has been described can be produced at once, as observed by Fedorov(1), by a drop of acetic or other acid of around pH 3.5. The effects do not reverse themselves. They are reversed by a drop of dilute alkali, however, which produces a pronounced opacity which gives way to translucency once more on reapplication of acid. The gradual enhancement of visibility without acid, in preparations untreated except with Ringer's, is presumably the result at least in part of accumulation of acid metabolites. The acidity would bring micelles of protoplasm closer to their isoelectric points, reducing their lyophilia and precipitating more of the coacervate out of the dispersion medium.

Functional Integrity and Morphologic State. Stimulation of the vagus is effective in diminishing or arresting the heart beat even when the visible end-feet are in the stage of slight enlargement, and ganglion cells mostly in the stage of enhanced visibility. In a subsequent stage of deterioration, the end-feet are very few and the cells are tenacious of supravital stains, which earlier they reject except for cytoplasmic granules. A functional integrity of synapses persists until most of the end-feet and cells are in these states of more advanced deterioration.

Microscopy of Synaptic Transmission. Changes in the appearance of the synapses can be observed under the microscope during tetanization of the vagus which results in inhibition of the heart beat. The visible changes are *not* instantaneous, but appear in from 5 seconds to a minute. The end-feet become more glistening and seem as if distended with or exuding clear liquid. They sometimes become yellowish, an effect which can sometimes also be produced by a drop of acetylcholine. The ganglion cells develop a greater clarity of detail. On cessation of the stimulation, the effects tend to subside partially or completely. The observations confirm in general and amplify those of Fedorov on the *excised* heart(1).

Summary. The synapses of beating, non-excised frog heart are amenable to microscopy with the vagi intact, and reveal a number of differences from the synapses of beating heart which has been excised. The terminals and end-feet of the vagi, and the ganglion cells on which they impinge, can be observed to pass through two stages of morphologic deterioration. The vagal effect can be produced until the second stage predominates in the preparation. Synaptic transmission from tetanization of the vagus is followed by visible changes in both the pre- and the postsynaptic structures.

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Growth of Dogs on Purified Diet Plus Aureomycin and/or Vit. B_{12}. (19637)

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The basal diets used in nearly all the published studies on the effect of antibiotics on growth were practical mixtures of grains, soybeans and other crude feeds. Only one or two reports(1) have indicated the effectiveness of the antibiotics in chicks when purified adequate diets were given. The question of the value of addition of antibiotics to complete rations, adequate for normal growth of young animals maintained under sanitary conditions and disease-free, has been raised by Speer *et al.*(2). A study of the mechanism of any growth stimulation obtained under these conditions might throw further light on the problem. The reasons usually suggested for the beneficial effects observed are the production of favorable, or inhibition of unfavorable intestinal microflora under the influence of the antibiotics. This may result in the prevention of loss of dietary vitamins present in marginal amounts or in the production of additional vitamins favorable to the host.

Since in the dog intestinal bacterial activity is apparently less intense and varied than in some other non-ruminants(3) a study of the effect of antibiotics on dog growth might be illuminating. Good growth can be obtained in dogs with rather simple purified diets and the performance of the strain of cocker spaniels maintained in this laboratory for several generations on these diets has been recorded (4). The experiment was set up therefore to determine whether an antibiotic, aureomycin, when added in crystalline form to such a purified complete diet could increase the rate of growth. Vitamin B_{12} was also given to a group of animals both with and without the aureomycin, although there was no indication that these dogs required such an addition to this diet. An attempt was also made to determine the composition of the growth.

Experimental procedure. Two litters of weanling cocker spaniel puppies from the laboratory colony, weighing 1.5 kg were maintained on the experimental diet for approximately 20 weeks. Each dog was caged individually. The dogs were weighed 3 times per week and the amount of food given daily was based on the body weight, 50 g per kg at first, and about 25 g at the end of the period. The composition of the diet, given in a previous publication(4), insured generous provision of protein, 20% vitamin-free casein, all B vitamins including 60 mg per kg per day of choline and 30 of inositol, but no vit. B_{12}, and all necessary fat-soluble vitamins. Four groups were formed and fed as follows: I. The basal diet only, II. The basal diet containing 5 μg crystalline vit. B_{12}* per 100 g, III. The basal diet containing 5 μg crystalline vit. B_{12} and 10 mg crystalline aureomycin† hydrochloride per 100 g, IV. The basal diet containing 10 mg crystalline aureomycin hydrochloride per 100 g. Blood was taken from the femoral vein or artery at 4, 7, 10, 13, and 16 week intervals for determination by the usual methods(4) of the hemoglobin, non-protein nitrogen and serum proteins content. Three nitrogen balance periods of 4 days each were done. Periodic measurements of skin fold thickness were made by shaving an area in the thoracic region of the back and measuring the skin fold with a mm micrometer. On sacrifice, a lethal dose of sodium nembutal was administered intravenously, and as soon as the animal was relaxed, the jugular vein was severed for complete bleeding. The viscera were removed and each organ weighed separately. All visible fatty tissue in the abdominal and thoracic cavities was removed, pooled and weighed. The fatty tissue was dried in a vacuum oven at 60°C to constant weight and the fat content determined by continuous Soxhlet extraction for 72 hours with petroleum ether (boiling point 30-60°). Specific gravity determinations on the eviscerated animal were made by the water displacement method of Rathbun and Pace(5), and the per cent body fat calculated by their formula proposed for the conversion of human body specific gravity to the corresponding fat percentage. These authors proposed 2 formulas, one applicable to guinea pigs that was developed on the basis of actual determinations, and one for humans derived from the guinea pig formula. The difference between the two lies in the value for the specific gravity of body fat characteristic of the species. Since the average value for the specific gravity of dog body fat based on determinations from 5 animals used in this study was identical with that found for humans, we felt justified in using the following formula of Rathbun and Pace for calculating the per cent body fat from specific gravity determinations: $\% \text{ fat} = 100 \left(\frac{5.548}{\text{Sp. gr.}} - 5.044 \right)$. The specific gravity of the fat of the 5 dogs was found to be 0.9164, 0.9150, 0.9210, 0.9206, 0.9120, mean 0.9170.

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* Crystalline vit. B_{12}. Lederle Laboratories Division, Cyanamid Co., Pearl River.

† Aureomycin HCl crystalline. Lederle Laboratories Division, American Cyanamid Co., Pearl River.

Results. No differences in hemoglobin, nonprotein nitrogen or serum protein levels were found among the 4 groups. As has often been noted the hemoglobin and serum protein levels increased steadily with age in all cases, with some tendency toward greater increases in the globulin than in the albumin fraction. All of these values were normal. The hemoglobin increased from 13% at 4 weeks on the diet to 15.5 at 16 weeks, and the serum protein from 4.4% at 4 weeks on the diet to 5.0 at 16 weeks. All of the dogs were in positive nitrogen balance, retaining on the average 651 mg per kg per day at 9 weeks on the diet, 447 at 13 weeks, and 348 at 16 weeks. No differences in intakes or retention were found among the diet groups and little variation in the individual animals.

Growth and body composition. There was a greater gain in weight shown by 2 of the 3 dogs fed vit. B_{12}, by 3 of the 4 given both vit. B_{12} and aureomycin, and by one of the 2 given aureomycin alone, than by the 2 control dogs. Unfortunately the sexes were not evenly divided among the groups but the normal performance of these young dogs has often been recorded. One animal in each of the 3 experimental groups lagged behind the others, although not behind the control group. The utilization of food for growth was slightly better in all the vit. B_{12} and aureomycin-fed dogs than in the controls, again excepting the same 3, which merely equalled the control performance (Table I). The eviscerated weights followed the same pattern, as did the carcass specific gravities. Three of the animals, 2 in the aureomycin plus vit. B_{12} group and one in the aureomycin group, were not sacrificed and these latter data are therefore not available for them. The 2 controls and the similar dogs given vit. B_{12} or aureomycin and vit. B_{12} had specific gravities ranging from 1.048 to 1.041, the other 4 overweight animals 1.031 to 1.013 (Table I).

When the carcass fat was calculated according to the formula of Rathbun and Pace(5) all of the experimental carcasses yielded higher value than did the 2 controls. Even dogs 5♂ and 8♀ which grew no better than the controls had slightly higher fat contents. The fat-free weights, moreover, of all but one of the dogs in the 3 experimental groups were no different from those of the controls. The one exception, 3♂, which had significantly greater fat-free weight than did the controls was the largest of the 11 dogs and the proportion of fat calculated for the live weight was the largest of the 8 animals sacrificed. The adipose tissue of the overgrown dogs was likewise richer in fat than in the controls and again this was true even though 2 of the former had no greater live weights than the latter.

The thickness of skin folds as suggested by Keys *et al.*(6) may be considered an indication of amount of subcutaneous fat. Only 3 of the dogs, 2 on vit. B_{12} and one on aureomycin, had obviously increased skin fold thickness over those of the controls. The heaviest dog, 3♂, showed normal fold thickness. For this group of dogs skin fold thickness did not correlate well with adiposity as measured by other means.

Discussion. Although the food intakes of these dogs were maintained equal in proportion to body weight and there was no difficulty of appetite in any case, the gain in weight varied from 18 to 25 g per 100 g food intake. Two of 3 dogs on the vit. B_{12} dose, 3 of 4 on

TABLE I. Growth and Body Composition of Young Dogs Fed a Purified Diet for 20 Weeks With and Without Vit. B_{12} and/or Aureomycin.

Diet, dog No., sex	Final live wt, kg	Total gain, kg	Gain/100 g food, g	Eviscerated wt, kg	Specific gravity	Carcass fat		Fat free wt, kg	Adipose tissue		Skin fold thickness, mm
						%	Total, kg		Wet wt, g	Fat, %	
Control											
1 ♀	6.3	4.3	18	4.9	1.048	25	1.57	4.7	145	76.5	4.3
9 ♀	6.7	4.5	19.2	5.3	1.042	28	1.88	4.8	162	59.7	5.5
Vit. B_{12}											
4 ♀	7.4	5.3	21.2	5.9	1.021	39	2.89	4.5	216	79.6	7
5 ♂	6.9	4.6	19.2	5.4	1.038	30.1	2.08	4.8	172	83.5	4.1
10 ♂	8.3	6.3	24.7	6.4	1.018	40.6	3.37	4.9	327	82.3	9.3
Vit. B_{12} + aureomycin											
2 ♀	8.3	6.5	24.1								
8 ♀	6.6	4.8	20.4	5.3	1.041	28.5	1.88	4.5	165	85.2	4.8
3 ♂	9.3	7.3	23.2	7.4	1.013	43.3	4.03	5.3	447	89.3	5.7
6 ♂	9	7.3	25								
Aureomycin											
11 ♀	7.6	5.5	20.5	6.1	1.031	33.7	2.56	5	294	85.2	8.5
7 ♀	6	4.2	18.1								

vit. B_{12} plus aureomycin, and one of 2 on aureomycin exceeded the control animals in total weight gain and in empty carcass weight. The specific gravity of the carcasses of these dogs was lower, the per cent carcass fat was higher, the adipose tissue richer in fat and in 3 cases the skin folds thicker than in the 2 controls and the 2 other dogs which resembled the controls in performance.

With such a small number of animals it is obvious that no certainty exists as to the reasons for these differences. In some cases vit. B_{12} and/or aureomycin caused better than usual feed utilization with production of extra body fat accounting for the weight additions. No corresponding increases in nitrogen retention were found nor was there any change in the composition of the nitrogenous blood constituents. If the intestinal microflora were involved in the extra fat deposition seen in these dogs it must have been through the stimulation by aureomycin of production of vit. B_{12} since one dog, 11♀, which received aureomycin but no vit. B_{12} accumulated extra fat. The other 5 overgrown dogs received vit. B_{12}. If this is true this is the first indication that young dogs fed a casein-rich diet may need dietary vit. B_{12}.

Determination of the composition of the increased growth produced by feeding antibiotics in chicks, turkeys and swine, not so far reported, is of obvious interest.

Summary. Eleven young cocker spaniels from 2 litters were fed from weaning for 20 weeks a purified complete diet which had been shown previously to produce optimum growth. Vit. B_{12} at the level of 5 μg %, aureomycin hydrochloride at the level of 10 mg % or both these substances were added to the diet of some of the animals. No differences resulted in nitrogen retention or in the nitrogenous constituents of the blood of any of the dogs. Two of the 3 dogs which received vit. B_{12}, 3 of the 4 which received both vit. B_{12} and aureomycin and one of the 2 which received only aureomycin gained more weight per 100 g food than did the 2 controls and the 3 others. These overgrown dogs had greater total gains, greater empty carcass weights, lower body specific gravity, more carcass fat, and more fat-rich adipose tissue than the 5 others. However, the fat-free carcasses were not significantly heavier. It is concluded that better food utilization may result in dogs in some cases from the addition of vit. B_{12} and/or aureomycin to a complete purified diet and that this growth is due solely to increased fat deposition.

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Complement-Fixing Murine Typhus Antibodies in Vitamin Deficiency States.* III. Riboflavin and Folic Acid Deficiencies. (19638)

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(Introduced by F. S. Cheever.)

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The purpose of this investigation, and others in progress, is an attempt to find an explanation for the increased susceptibility and mortality that occurs in malnourished animal and human populations exposed to typhus(1) and other rickettsial and bacterial infections. Wertman and Sarandria(2) reported that pantothenic acid and thiamine deficiencies influenced the antibody response when a relatively small amount of immunizing material was injected. However, the influence was not as apparent when relatively large amounts of the antigenic material was injected. In addition, they also reported(3) a severe impairment of antibody response in pyridoxine deficient rats when a rickettsial antigen (*Rickettsia typhi*) containing 0.0073 mg N was injected. However, rats deficient in nicotinic acid did respond and produce complement-fixing antibodies when injected with the same amount of antigen. Stoerk, Eisen, and John(4) reported normal serum antibody titers in thiamin, riboflavin and pantothenic acid deficiencies. However, Axelrod *et al.*(5) noted a greatly decreased titer in pantothenic acid, pyridoxine deficiencies and variable but lower titers in riboflavin deficiencies. Ludovici and Axelrod(6) reported severe impairment of antibody response in the pteroylglutamic acid-deficient rats and a moderate impairment in vit. A and niacin-tryptophan deficiencies. These investigators employed a 10% suspension of washed Group O, Rh positive human erythrocytes in physiological saline for antibody stimulation and measured the hemagglutinin production. The present paper deals with the effects of riboflavin, and folic acid deficiencies upon complement-fixing antibody production under similar experimental conditions(2,3).

Experimental. Male weanling albino rats of the Sprague-Dawley strain, distributed into groups as indicated in Table I, were housed individually in wide-meshed, screen-bottom cages and weighed at regular intervals. The ingredients used in preparing the different diets were obtained from General Biochemicals, Inc., and Nutritional Biochemicals Co. All ingredients were of the highest purity obtainable and each basal diet was formulated so that only a minimum amount of the B complex vitamins to be studied were present. The basal diet for the riboflavin groups was as follows: Sucrose, 58.75%; casein (vitamin-free), 25.00%; vegetable oil (hydrogenated), 10.00%; salt mixture No. 2 (U.S.P.), 4.00%; corn oil (Mazola), 2.00%; choline chloride, 0.20%; i-inositol, 0.03%; paraminobenzoic acid, 0.01%; dl-alpha-tocopherol acetate, 0.01%; and 2-methyl-1,4-naphthoquinone, 0.001%. The basal diet for the folic acid groups was as follows: Sucrose, 57.77%;

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TABLE I. Complement-Fixation Titers and Body Weight Change of Rats in Riboflavin and Folic Acid Deficiency States.

Diet and immunization	No. rats	Complement-fixation titers									Body wt	
		0	1:10	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	Initial avg, g	Final avg, g
Riboflavin deficient	26											
1 inj.		26	—	—	—	—	—	—	—	—		
3 "	*	—	—	—	1	1	2	11	8	1	51	93
Riboflavin control (paired weighed)	10											
1 inj.		1	1	1	4	2	1	—	—	—		
3 "		—	—	—	—	—	—	—	6	4	49	92
Riboflavin control (*Ad lib.*)	9											
1 inj.		5	1	1	2	—	—	—	—	—		
3 "		—	—	—	—	—	—	4	2	3	53	296
Folic acid deficient	25											
1 inj.		25	—	—	—	—	—	—	—	—		
3 "	†	1	—	1	6	8	1	—	1	—	39	126
Folic acid control (paired weighed)	10											
1 inj.		2	4	3	1	—	—	—	—	—		
3 "		—	—	—	—	—	—	1	4	5	38	121
Folic acid control (*Ad lib.*)	10											
1 inj.		3	2	4	1	—	—	—	—	—		
3 "		—	—	—	—	—	—	3	3	4	38	311

* 2 rats died. † 7 rats died.

casein (vitamin-free), 25.00%; vegetable oil (hydrogenated), 10.00%; salt mixture No. 2 (U.S.P.), 4.00%; corn oil (Mazola), 2.00%; sulfasuxidine (succinylsulfathiazole), 1.00%; choline chloride, 0.20%; i-inositol, 0.03%; dl-alpha-tocopherol acetate, 0.01%, and 2-methyl-1,4-napthoquinone, 0.001%. In addition, 2 drops of Haliver oil (3,000 U.S.P. of vit. A and 24 U.S.P. of vit. D) were given by dropper to each rat once a week. The rats in the riboflavin and folic acid groups received their supplemental vitamins daily in the form of a pill. The riboflavin-deficient group had riboflavin omitted from the pill and the folic acid-deficient group had folic acid omitted. Each pill for the riboflavin-deficient group was compounded to supply the following vitamins (7): Thiamine, 40 γ; pyridoxine, 50 γ; calcium pantothenate, 150 γ; nicotinic acid, 150 γ; biotin, 1 γ; folic acid, 1 γ. Each pill for the folic acid-deficient group was compounded to supply the following vitamins(6,7): Thiamine, 40 γ; riboflavin, 60 γ; pyridoxine, 50 γ; calcium pantothenate, 300 γ; nicotinic acid 150 γ; and biotin, 3 γ. The folic acid control group received daily 20 γ of folic acid in the vitamin pill daily and the riboflavin control group received 60 γ of riboflavin in addition to the daily vitamin supplement made available to the deficient animals. In addition to the control groups that were given the complete basal diet plus the entire vit. B requirement, inanition control groups were included to determine the influence of a reduced food intake upon antibody production when all the necessary B vitamins were present. Ten animals for each group were paired-weighed against 10 rats in the deficient groups. At the end of the period necessary to establish the deficiency, each rat was injected intraperitoneally with 0.5 cc (0.0073 mg N)[†] of washed, formalinized suspensions of *Rickettsia typhi* (murine typhus).[‡] One cc inoculations were repeated at 7-day intervals until each rat had received a total of 2.5 cc (0.0365 mg N). During this period, the animals were bled 7 days after inoculations of murine typhus vaccine to determine if the amount of antigenic stimulus played a role. The complement-fixation technic employed in this study was identical to that of Plotz(8), Plotz and Wertman(9), and Wertman(10). Fixation was allowed to take place for 18 hours at 4 to 6°C. The sensitized cells (0.25 ml sheep cells 3% and 0.25 ml amboceptor containing 3 MHD) were added and the tubes incubated for 30 minutes at 37°C in a water bath. The tests were read following secondary incubation and only 4^+ and 3^+ fixations were accepted as end-points. The controls necessary to insure valid results and reproducibility were included in all the titrations and serum dilution tests.

[†] Nitrogen content determined by Biophysics Department, University of Pittsburgh.

[‡] Purified antigens supplied by Dr. Herald Cox, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

Results. The riboflavin and folic acid control rats at the end of the feeding period, all had gained weight, appeared healthy and possessed smooth even coats. The rats fed on the riboflavin and folic acid diets showed the signs which might be expected to accompany their respective deficiency. The inanition control groups showed approximately the same weight gain as their respective deficiency groups. The initial average and final average weight of each group are recorded in Table I. The riboflavin deficient group and the folic acid deficient group gained much less weight than their respective control group.

The table demonstrates the end-point titers obtained in the complement-fixation test using a suspension of washed murine typhus rickettsiae as the antigen. It appears that both riboflavin and folic acid deficiencies interfere with the production of circulating antibody when a relatively small amount of antigenic material was injected. None of the animals in these 2 groups demonstrated detectable amounts of complement-fixing antibodies. However, the sera of the inanition (paired-weighed) controls and the sera of the *ad libitum* controls demonstrated antibody formation in approximately 75% of the animals. The riboflavin deficient rats and the folic acid deficient rats both demonstrated antibody production after the introduction of more antigenic material (0.0365 mg N). However, the folic acid deficient rats produced less antibody than either the riboflavin deficient or control group. An average quantitative difference of approximately 3 two-fold dilutions existed between the folic acid deficient and folic acid control group even when relatively large amounts of murine rickettsiae were injected. There was an average difference of approximately one two-fold dilution between the riboflavin deficient and the riboflavin control group. It is evident that the reduced food intake alone did not appreciably influence antibody production as measured by complement-fixation.

Summary. 1. It appears as though the riboflavin requirement for antibody production is not as critical as the folic acid requirement. 2. This study indicates that both riboflavin and folic acid deficiencies impaired the production of circulating complement-fixing antibodies when relatively small amounts of antigen were injected. However, after more antigen was introduced, significant titers were obtained from the sera of riboflavin deficient rats but the folic acid deficient animals produced significantly less antibody. The inanition control (paired-weighed) and the normal control animals (fed *ad libitum*) demonstrated antibody production of approximately the same titers when injected with either 0.0073 mg N or 0.0365 mg N. Therefore, it appears as though the specific vitamin deficiency and not the inanition is the significant factor in the production of circulating complement-fixing antibody.

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Effect of X-Irradiation upon the Enzyme Systems of the Mouse Spleen.
(19639)

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Barron, employing tissue slices, reported that the respiration of most tissues and the oxidation of substrates requiring sulfhydryl enzymes was diminished immediately after irradiation, with the thymus as the only exception(1,2). LeMay, using the homogenate technic, found the succinoxidase system of the rat kidney (a sulfhydryl enzyme system) to be unaffected by 400-800 r(3). Dubois *et al.* have demonstrated that cytochrome oxidase and succinic, malic and triosephosphate dehydrogenase are all unaffected by lethal whole body irradiation, and that certain alkaline phosphatases are increased so that liver, kidney and thymus homogenates have a decreased ability to esterify phosphorus or, at least, to maintain it in the form of its high energy esters(4). They were, however, unable to show any effect upon the adenosinetriphosphatase (ATPase) content of the tissues studied.

Recent reports from Jacobson's laboratory (5,6) have centered interest on the role of the spleen in radiation trauma and prompted further study of the biochemical properties of that organ. The present work was undertaken to evaluate the effect of whole body irradiation upon the succinoxidase, alkaline phosphatase and aerobic phosphorylation systems of the mouse spleen.

Methods. The animals used in these experiments were 6-7-week-old white male mice of the NIH brother-sister strain. The radiation factors were 200 KV, 20 ma, 0.51 mm aluminum and 0.25 copper added filtration, 50 cm target distance and a dose rate of 62-66 r per minute. Ten mice were irradiated together in a perforated plastic container 2.5 cm deep, 15 cm in diameter with walls 1.5 mm thick. The animals were given a total exposure of 640 r which has been shown to be an LD_{90} dose in this laboratory(7). The analyses for the *succinoxidase*(8) and *aerobic phosphorylation*(9) systems were carried out in the conventional Warburg apparatus at 37°C with air as the gas phase. The Na β glycerophosphatase(10) 5-nucleotidase(11) and ATPase (8) systems were incubated in centrifuge tubes for 15-30 minutes at 37°C, precipitated with trichloracetic acid and the phosphorus analysis carried out on the supernatant according to the method of Fiske and Subbarow. The hexokinase, prepared from bakers yeast according to Berger, Colowick, Slein and Cori (12), was brought to step 3A and then lyophilized in a 1% glucose solution.

Respiratory enzymes. It was necessary in this, as in the following experiments, to pool the spleens of several animals in order to have sufficient tissue for analysis, more spleens of irradiated animals being required because of the weight loss from a normal average of 110 mg to 33 mg. The homogenates were prepared in a Potter-Elvehjem type homogenizer as a 10% suspension in cold distilled water and were used immediately. The assays were carried out at two or more levels of tissue concentration and the activity was found to be strictly proportional. For the succinic dehydrogenase system 0.2 and 0.4 cc of the 10% suspension were used and for the cytochrome oxidase 0.10, 0.15 and 0.20 cc of a 5% suspension. Assays were run at intervals from 4 hours to 17 days after irradiation simultaneously with the non-irradiated controls. As shown in Table I, there was no effect of irradiation upon the activity of either the succinic dehydrogenase or the cytochrome oxidase in the mouse spleen on the basis of equal dry tissue weight, which agrees with the results of others on liver, kidney and thymus. Because of the loss in weight of the spleens from the irradiated animals, however, it is clear that the absolute quantities of both enzymes are reduced in exact proportion to the weight loss—an average of about 70%.

TABLE I. Effect of X-Irradiation on Succinoxidase System of Mouse Spleen Homogenates. Values expressed as mm^3 O_2/mg dry wt/hour.

No. of assays*	Succinic dehydrogenase Control	Succinic dehydrogenase Irradiated	Cytochrome oxidase Control	Cytochrome oxidase Irradiated	Avg spleen wet wt (mg) Control	Avg spleen wet wt (mg) Irradiated†
21	21.6 ± .4	20.6 ± 2.26	110 ± 6.7	116 ± 3.1	110 ± 6.1	33 ± 2.1

* Assays from 4 hr through 17 days after irradiation with controls run simultaneously.
† Average of 100 spleens from 24 hr after irradiation through 14 days.

TABLE II. Effect of X-Irradiation on Alkaline Phosphatases of Mouse Spleen Homogenates. Values expressed as μg phosphorus liberated/mg nitrogen/hr.

Time after irradiation	ATPase Control	ATPase Irrad.	5-Nucleotidase Control	5-Nucleotidase Irrad.	β-glycerophosphatase Control	β-glycerophosphatase Irrad.	Avg spleen wet wt (mg) Control	Avg spleen wet wt (mg) Irrad.
4 hr	2180	2430	115	135	33.6	30.7	115	112
1–11 days*	2068±89	7120±455	106±3.6	201±30	26.3±1.4	54.1±3	139±3	40±3.4

* Figures are averages from 8 assays beginning 1 day after irradiation and continuing through 11th day.

II. *Alkaline phosphatases.* Since the activity of the mouse spleen was markedly different towards all 3 of the various substrates chosen, slight modifications of the described procedures had to be used. Thus, with Na β glycerophosphatase the test system contained 0.8 cc of a 0.025 M barbital buffer (pH = 8.9); 0.2 cc of 0.015 M Na β glycerophosphate, 0.1-0.2 cc of 3% aqueous homogenate and water to make 1.6 cc. The incubation period was 30 minutes at 37°C. The *5-nucleotidase* system contained 0.35 cc of a 0.025 M barbital buffer (pH = 8.9); 0.05 cc of 0.04 M $MgCl_2$; 0.15 cc of 0.015 M adenylic acid (adjusted to pH = 8.9); 0.1-0.2 cc of a 3% aqueous homogenate and water to make 0.8 cc. The incubation period was 30 minutes at 37°C. The *ATPase system** contained 0.4 cc of 0.05 M barbital buffer (pH = 7.4); 0.05 cc of 0.04 M $CaCl_2$; 0.15 cc of 0.013 M ATP, 0.1-0.2 cc of a 1% aqueous homogenate and water to make 0.8 cc. The incubation period was 15 minutes at 37°C.

Controls for substrate and tissue were run with each determination and the appropriate corrections made. The above systems were found to be proportional to the tissue concentrations at the indicated levels. The values were calculated on both a total nitrogen and a total phosphorus basis, the latter showing the same general picture as the former.

As can be seen from Table II, there was after irradiation a sharp increase in activity of all the phosphatases. This was particularly noticeable in the case of ATP which is in marked variance with that found in other tissues and represents an interesting and perhaps significant tissue specificity. It is important to realize that, although the activity of these phosphatases increased sharply, the total content per spleen decreased due to the marked involution of this organ. Thus, the glycerophosphatase and 5-nucleotidase lost approximately 1/3 to 1/2 of their absolute value; the ATPase remained almost constant.

Aerobic phosphorylation. For these determinations the inorganic phosphorus content per flask was dropped to 0.3 cc of 0.011 M phosphate (103 γ P per flask) and the amount of tissue used was 0.6 cc of a 10% isotonic KCl homogenate. It was found that the spleen is deficient in creatine transphosphorylase and consequently it was impossible to tap off the high energy phosphate as the creatine ester. However, there was a significant uptake of inorganic phosphorus which was unaffected by the presence of creatine at a concentration of 0-30 mg per flask.

It could be shown (Table III) that the disappearance of inorganic phosphorus was dependent upon the oxidative process, for when succinate was omitted there was a marked increase in the inorganic phosphorus, presum-

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* Newer developments in this system as reported by V. R. Potter and H. C. Simonson, *Fed. Proc.*, 1952, v11, 270, indicate serious difficulties in interpretation of the "Standard" ATPase Assay. Further work in progress to clarify this situation.

TABLE III. Effect of X-Irradiation upon Aerobic Phosphorylation in Mouse Spleen Homogenates.

	O_2 uptake, mm^3/mg N/10 min		Phosphorus uptake, μg P/mg N/20 min; control spleen			Phosphorus uptake, μg P/mg N/20 min; irradiated spleen			
Time after irradiation	Control spleen	Irrad. spleen	With succinate	Without succinate	Total μg P esterified	With succinate	Without succinate	Total μg P esterified	% loss in overall esterification
4 hr	11.6	10.1	+31	–20	51	+10	–22	32	37
1 day	11	12.1	+30	–26	56	–12	–46	36	36
2–11 days*	11.9±.5	12±.5	+25±1.5	–26.5±1.6	51.7±1.5	–37±4.1	–58±3.9	21.4±2.9	56.4±7.3

* Figures are averages from 8 assays beginning 2 days after irradiation and continuing through 11th day.

ably due to the splitting of the ATP even though the system contained NaF. Thus, the algebraic sum of the changes in the flask with and without substrate represents a net phosphorus uptake against a constant dephosphorylating gradient.

After irradiation, the ability of the spleen homogenate to esterify P, or to maintain it in a high energy state, is strongly impaired.† Whether this is due to the post irradiation rise in ATPase as shown in the preceding section or whether there is a more direct effect of radiation upon the transphosphorylation process cannot be answered by this experiment. That the oxidation of the substrate in this system was essentially unaffected after irradiation would be expected on the basis of the earlier results with the succinoxidase system. Therefore, it is clear that any decrease in phosphorylation cannot be assigned to deficiencies in the oxidation of succinic acid but must be linked with one of the subsequent steps.

Several experiments were run in which hexokinase and glucose were added to the experimental flasks. This procedure increased considerably the net esterification and it was possible to show that all the inorganic phosphorus which disappeared was present as glucose-6-phosphate. This system shows exactly the same phenomenon as the above in which the intermediate phosphate esters were not identified.

Discussion. It is clear that spleen homogenates reveal the same general metabolic response pattern to irradiation as do other tissues, insofar as the respiratory enzymes and aerobic phosphorylation are concerned. The one major point of difference revealed so far is the marked increase in the ability of the irradiated spleen homogenate to break down ATP. Whether this be the cause for the decreased ability of the irradiated spleen to perform coupled phosphorylation or not cannot be answered as yet. Further work is in progress in an attempt to evaluate the significance of this phenomenon.

Conclusions. Total whole body irradiation of white mice with 640 r (L_{90} dose) revealed the following changes in the metabolism of spleen homogenates: 1) no effect upon the activity of the succinoxidase system, 2) marked increase in activity of alkaline phosphatase which was especially marked with ATP as substrate and 3) decreased ability to synthesize (or maintain) the high energy phosphorus compounds coupled with the oxidation of succinate.

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Studies on Chick Embryo Adapted Rabies Virus. III. Duration of Immunity in Vaccinated Dogs. (19640)

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Although attempts have been made to immunize dogs against rabies as far back as 1826(1) and Pasteur succeeded in doing so in 1885(2), only few extensive studies have been conducted on duration of immunity following vaccination of dogs against this disease. A summary of these studies is shown in Table I. Pasteur reported(2) that resistance to intracerebral infection still appeared to be manifest 2 years after the "immunizing" injection. Data obtained by other investigators and summarized by Schnürer(3) seemed to indicate that immunity can be observed even 363 days after vaccination with living virus, although lack of adequate controls makes interpretation of their data more difficult. In the experiments of Miessner and Baars(4), dogs were found to be solidly immune to challenge inoculation with street virus 8 to 14 months after vaccination with fixed virus vaccine. More recently, a series of experimental studies designed to determine the duration of immunity following vaccination of dogs has been published by Johnson(5), and these are also summarized in Table I. It may be observed that preparations of phenolized rabies vaccine conferred an effective immunity upon dogs challenged with street virus one year after vaccination. Of those animals immunized with a single injection of vaccine, 11.5% died after challenge inoculation, as compared with 79% of the controls. Conversely, if the vaccination process consisted of 3 injections given at weekly intervals, none of the vaccinated dogs came down with rabies after challenge one month later, in contrast with 68% which died in the control group.

In view of the fact that an effective single injection method of canine vaccination against rabies was found in the recently developed chick embryo vaccine containing the living Flury strain of rabies(6), it seemed to be of interest to determine the duration of immunity conferred upon dogs by inoculation with this vaccine.

Materials and methods. Virus strains. The Flury egg-adapted strain of rabies virus used in this study has been described(7). An infected chick embryo suspension desiccated from the frozen state was used for vaccination of dogs. It represented the 40th egg passage of the virus, and was prepared 8 months prior to vaccination. The PM (Pitman-Moore 980) rabbit-fixed strain of virus(8) was used for the preparation of phenolized vaccine of horse brain origin. This particular lot of vaccine, bearing Lab. No. 6273-1580, was prepared on April 13, 1948, and the Habel mouse potency test was performed on May 4, 1948, with results indicating that the vaccine protected against 8166 LD_{50} of fixed virus. For challenge purposes the NYC strain of rabies street virus representing either the 6th or 7th passage canine salivary glands in this laboratory was used on the vaccinated animals. The preparation of the challenge inoculum followed that described in detail elsewhere(6). *Animals.* Mongrel dogs over 6 months old

TABLE I. Some Observations on Duration of Immunity Following Vaccination of Dogs Against Rabies.

Immunization		Time interval between immunization and challenge	Challenge inoculation		Mortality ratio		Author
Virus	Route		Virus	Route	Vaccinated	Controls	
Fixed or Street	Subcut.	1 yr	Street	Intracer.	3/14	?	Pasteur(2)
		2 "	"	"	2/6	?	
Fixed	Subcut. or intraper.	18–120 days	Fixed	"	3/18	?	Summarized by Schnürer (3)
			Street	"	8/22	?	
		4–363 "	Same	Intraocular	2/24	?	
				"	1/10	?	
		23– 56 "	Same	Intramusc.	0/2	?	
				"	0/14	?	
		15– 62 "	Street	Bite	0/3	?	
Fixed	Intraper.	8– 14 mo	"	Intramusc.	0/9	3/3	Miessner & Baars(4)
Phenolized vaccine	Subcut.*	1 yr	"	Intramasseter	6/52	41/52	Johnson(5)
	" †	1 mo	"	"	0/25	17/25	

* One inj. of phenolized vaccine. † Three inj. of phenolized vaccines.

were used in the experiments. The animals were kept under observation for a period of 3 months prior to vaccination. Following challenge inoculation, the animals were kept in individual cages throughout an observation period of 1½ years. An autopsy was performed on every animal that died and portions of the brain tissues and of the submaxillary salivary glands were removed. Impression smears of Ammon's horn of each dog were examined for presence of Negri bodies. In addition, 10% suspensions of brain and of salivary gland tissues were each inoculated intracerebrally into six 28- to 35-day-old Swiss albino mice, which were observed for a period of 3 weeks. In case of doubt as to the cause of death, brain tissues from such mice were subinoculated into 6 other mice. If the latter died as a result of infection, neutralization tests were performed using immune rabbit serum to determine whether the agent was rabies virus. All material containing living virus used in the experiments was titrated intracerebrally in 28- to 35-day-old mice; each of 6 mice received .03 ml of a given dilution. *Sera.* All nonvaccinated control animals were bled prior to challenge inoculation, and their sera immediately tested in order to exclude those animals whose blood showed neutralizing antibodies. In several instances, serum was also obtained from dogs prior to vaccination with either Flury or phenolized vaccines, and then at certain regular intervals prior to challenge inoculation. *Neutralization tests.* Undiluted sera were mixed in equal volume with a dilution of guinea pig brain suspension of the CVS rabbit-fixed strain which contained approximately 25-50 LD_{50} of the virus. The serum virus mixtures were incubated for 90 minutes at 37°C before being injected intracerebrally in Swiss albino mice.

Experimental. On Feb. 8, 1949, 120 dogs which had been under observation for 3 months were divided into 2 equal groups. One group of animals was immunized by a single injection in the upper quadrant of the posterior surface of the thigh muscle with 5 ml of a 20% suspension of chick embryos infected with the 40th egg passage of the Flury strain. This material was rehydrated at the time of vaccination and kept in iced water during the inoculation period. Simultaneously titrated in mice, it had an LD_{50} of $10^{-3.99}$. The other group of 60 animals received a divided dose of 5 ml of a 20% suspension of horse brain containing phenol-inactivated rabies virus, 2½ ml being administered in each of 2 sites. During the ensuing 2 years all animals were closely observed, and while some losses occurred in both groups as a result of fights or intercurrent infections, in not a single instance could death of an animal be attributed to ill

TABLE II. Results of Challenge Inoculation with Street Virus of Dogs Vaccinated with Rabies Vaccines.

Interval between vaccination and challenge	Vaccine	Results of challenge					
		No. of dogs				Rabies virus isolated from	
		Inoculated	Dead	% dead	Day of death after challenge	Brain tissue	Salivary gland
1 yr	Flury strain living virus	25	0	0		—	—
	Phenolized horse brain origin	22	3	14	18, 21, 260	3	1
	Nonvaccinated controls	25	18	72	15, 18, 18, 18, 18, 18, 18, 21, 23, 25, 25, 25, 26, 27, 29, 29, 33, 33	18	16
2 yr	Flury strain living virus	25	3	12	21, 22, 24	3	0
	Phenolized horse brain origin	19	8	42	14, 16, 17, 18, 18, 19, 20, 33	6	0
	Nonvaccinated controls	23	21	91	13, 15, 16, 16, 16, 17, 17, 17, 17, 18, 18, 18, 19, 20, 21, 22, 22, 22, 22, 22, 23	21	11

effects of the chick embryo vaccine. On the other hand, out of the 60 animals injected with the phenolized rabies vaccine, one died 16 and another 24 days following vaccination. Both deaths were preceded by paralysis and were found to have been caused by CNS demyelination characteristic of that seen following inoculation with material containing nervous tissue(9).

One year after vaccination, one-half of the surviving dogs in the groups immunized with the Flury strain or the phenolized vaccine were challenged with a 1:90 dilution of canine salivary gland infected with the 6th passage NYC strain of street virus. This preparation, titrated in mice, had a $10^{-4.85}$ LD$_{50}$ titer. The challenge dose consisted of a total of 0.2 ml injected bilaterally into the masseter muscle, 0.1 ml into each site. Inoculation was performed with a 1 cc tuberculin syringe equipped with a 1-inch, 20-gauge needle. The inoculum was deposited in 3 different sites next to the periosteum in order to assure better tissue penetration of the virus. As mentioned previously, the challenged dogs were observed for 18 months following inoculation. The remaining groups of vaccinated animals were challenged 2 years after vaccination with 1:75 dilution of canine salivary gland representing the 7th passage of the NYC strain of street virus. This time, the LD$_{50}$ titer of the challenge virus in mice was found to be $10^{-6.50}$.

The results of the 2 experiments are summarized in Table II. It may be observed that in the one-year challenge experiment, 18 out of 25 non-vaccinated controls died after exhibiting signs of clinical rabies, and the challenge virus was isolated from the brain tissue of all of the 18 animals and from the salivary gland tissue of 16 out of the 18. In contrast, none of the dogs with the Flury strain vaccine succumbed to rabies following challenge inoculation. Out of 22 dogs which were vaccinated with phenolized vaccine, 3 died following challenge, and challenge virus was isolated from the brain tissue of 3 animals and from the salivary gland tissue of one. The latter isolation occurred in a dog which died 260 days after challenge inoculation. Since this animal had been confined during the entire observation period in a separate cage with no contact with any other infected animals, his death must be attributed to the original challenge inoculation even though the incubation period was prolonged.

The results of challenge inoculation 2 years after vaccination show that 21 out of 23 non-vaccinated control animals died following rabies injection. Virus was isolated from the brain tissue of 21 animals but from the salivary gland tissue of only 11 dogs. Out of

TABLE III. Results of Neutralization Test on Sera of Vaccinated Dogs Challenged 1 Year after Vaccination.

Vaccine	Dog	Survival ratio of mice inoculated with virus and serum bleedings					Results of challenge with street virus*
		A	B	C	D	E	
Flury	A	0/5	5/5	—	5/5	4/5	S
	B	0/5	0/5	0/5	0/5	0/5	S
	C	0/5	4/5	4/5	5/5	3/4	S
	D	0/5	4/5	2/5	3/5	5/5	S
	E	0/5	5/5	4/5	4/5	3/5	S
	F	0/5	5/5	4/5	1/5	5/5	S
Phenolized	G	0/5	4/5	5/5	0/5	0/5	D
	H	0/5	0/5	1/5	0/5	0/5	S
	I	0/5	5/5	5/5	0/5	0/5	S
	J	0/5	0/5	0/5	0/5	0/5	D
	K	0/5	5/5	5/5	5/5	2/5	S
	L	0/5	4/5	2/5	—	0/5	S

* One year after vaccination.

A—Prevaccination bleeding; B—1 mo after vaccination; C—3 mo after vaccination; D—6 mo after vaccination; E—9 mo after vaccination. S—survived; D—died.

25 animals vaccinated with the Flury strain, 3 died following challenge, and virus was isolated from the brain tissue of these 3 animals but not from the salivary gland tissue of any of the dogs. Out of 19 animals vaccinated with phenolized vaccine, 8 died following challenge inoculation, and rabies virus was isolated from the brain tissue of 6 of the dead animals. However, the 8 dead animals in this group were all included as having died of rabies since clinical signs of either paralytic or furious rabies were observed in all 8 dogs. Perhaps the absence of the virus in the central nervous system tissue of the 2 animals may be ascribed to the phenomenon of "self-sterilizing neuro-infection"(10). Although evidence obtained in a previous communication(8) indicated that it is difficult to correlate serological evidence of immunity with the resistance of the vaccinated animal to experimental infection with rabies virus, another attempt was made in the present study to determine the presence of homologous antibodies in serum in dogs vaccinated with the 2 types of experimental vaccines.

In Table III are summarized the results of neutralization tests on sera of 6 dogs in each vaccinated group challenged one year after immunization. It may be observed that out of 6 animals vaccinated with the Flury strain, none had neutralizing antibodies prior to vaccination, and in all but one animal, immunization with the Flury strain elicited homologous antibodies. Interestingly enough, even this animal (dog B), which showed no neutralizing antibodies at any time after vaccination, survived challenge with street virus. Out of 6 animals inoculated with the phenolized vaccine, 2 did not develop neutralizing antibodies following vaccination; whereas in the remaining 4, a positive serological response was noted. This response, however, seemed to fade out after 9 months following vaccination. The results of challenge inoculation seemed again to corroborate previous evidence that serological response following immunization against rabies cannot be regularly correlated with the actual resistance of the animal to experimental infection with street virus.

In view of the above results, no further attempts were made to bleed vaccinated animals to determine the presence or absence of neutralizing antibodies in their sera. However, the control dogs which accompanied those challenged 2 years after vaccination were bled and their serum was submitted to neutralization tests. Every animal whose serum had neutralizing antibodies was deleted from the control group.

Comment. The difference between the challenge results obtained in the one-year and two-year immunity tests suggests a decline in specific resistance to rabies during the second year after vaccination. However, this decline may be more apparent than real. It will be noted that the challenge inoculum employed in the one-year test had a mouse LD_{50} titer of $10^{-4.85}$, while that used in the 2-year test was $10^{-6.5}$. Calculated on the basis of these mouse titers, the dogs in the one-year test were challenged with 5,194 LD_{50}'s and those in the 2-year test received 278,276 LD_{50}'s. This relatively heavier challenge is reflected in the 91% mortality and the 18.4 day average incubation period in the non-vaccinated controls on the 2-year tests, as compared with 72% mortality and 23.2 day average incubation period for the one-year test. Had challenge virus of the same potency as that used in the one-year test been employed in the 2-year challenge, the degree of protection shown in the 2-year test might have been enhanced.

In any attempt to translate the results of these laboratory experiments in terms of the protection afforded dogs against field exposure, it should be borne in mind that the amount of virus introduced by natural exposure is usually greatly exceeded by that employed in the laboratory challenge tests. Thus, if one may assume that the death of 3 dogs out of 25 challenged with street virus 2 years after vaccination with Flury strain was conditioned by the excessive dosage of the challenge virus, then it may perhaps be permissible to state that for all practical purposes vaccination with Flury strain confers immunity for at least 2 years.

Conversely, study of the trend of results of challenge inoculation of animals vaccinated with phenolized vaccine does not permit the drawing of as favorable a conclusion as in the case of vaccination with Flury virus. Although the phenolized vaccine certainly proved effective when dogs were challenged one year after vaccination, the death of 8 animals out of 19 challenged 2 years after vaccination seems to indicate that inoculation with phenolized vaccine constitutes a less effective method of protection than with the Flury strain. This statement may be supported by a statistical analysis of the results. By calculation of the exact probabilities, and by combination of these probabilities for the one- and 2-year results,* using the method of R. A. Fisher(11), one obtains a value of X^2 of 10.166 with 4DF corresponding to a probability of 0.038. The latter figure is lower than 0.05 and therefore indicates a statistically significant difference in favor of the immunizing power of the Flury strain vaccine as compared with the phenolized vaccine.†

Mass field vaccination of the canine population with living fixed strain of rabies virus (rabbit brain origin) has been attempted only once in the past, so far as we know, and with excellent results. Hindersson(12) immunized 2,908 dogs in the Nykyrka, Rautu, Salmis, and Suistamo regions of Finland beginning in 1929 through 1932, and by this means was actually able to eradicate rabies in the entire region of Finland which borders on Soviet Russia. He later observed the first, and as far as can be determined, the only case of rabies in the immunized group 2 years and 3 months after vaccination.

Conclusions. Hindersson's observations, coupled with the results of the present experiments, seem to indicate that: 1. Vaccination with living rabies virus is perhaps a more effective method of immunization of the canine population than any of the others presently available. 2. Immunity conferred by vaccination with living rabies virus, as exemplified by Flury strain in these experiments, lasts at least 2 years. 3. These observations on the duration of immunity following live virus vaccination indicate the need for reconsidering local ordinance and other regulations concerning rabies vaccination.

The authors wish to express their appreciation and thanks to Mr. George Lent for his excellent care and handling of the animals during these experiments.

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* This seems to be particularly permissible since animals in both experiments were vaccinated on the same day with the same material.

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Effects of Aureomycin on Renal Lesions, Liver Lipid, and Tissue Choline in Choline Deficiency. (19641)

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The impression was obtained in a previous study(1) that it might be possible, by causing certain alterations in the conditions in the gut, to reduce considerably the damage produced in rats by diets deficient in choline. Succinylsulfathiazole was studied at that time, but it was not observed to be effective in modifying the damage, although it did produce enlargement of the caeca. More recently, with the same idea in mind, the newer antibiotics have been examined. The present report presents evidence that the severe renal injury which occurred regularly as a result of choline deficiency was prevented in most cases by aureomycin. Fatty changes in the liver also appeared to be diminished somewhat and alterations in tissue and fecal choline were observed. However, it was not demonstrated conclusively that these effects of aureomycin were actually results of changing the conditions in the gut and not results of a direct metabolic effect.

Methods and materials. The animals employed were male rats of a Sherman strain, which were 20 to 23 days old and weighed 30 to 40 g when started on the experimental diets. The basal diet was the same as that described previously(1) with the carbohydrate in the form of sucrose, and with Wesson's modification of the Osborne-Mendel salt mixture with Zn and Co added in most experiments. The unsupplemented diet contained negligible amounts of choline, and presumably contained relatively small amounts of vit. B_{12}. The animals were maintained in individual cages with large-mesh screen bottoms, in a room with controlled temperature. The diets were fed *ad libitum* in some experiments. In others, each animal received 3.6 or 5.0 g of food each afternoon. In the latter experiments, the food was consumed completely and the food intake of all animals was the same. The supplements under study were incorporated in the diet in most cases in the form of pure, crystalline substances. After 10 days on the diets the surviving animals were autopsied after decapitation, and the results evaluated. It had been observed previously that few additional animals developed renal lesions or died after this period. In 4 experiments with 8 animals in each group, employing controlled feeding, total liver lipid, after 4 days on the diets, was determined gravimetrically as the petroleum ether-soluble portion of the material obtained by wet alcohol-ether extraction of homogenized tissue. Choline was determined by the method of Glick(2), either directly on the material extracted by alcohol-

TABLE I. Effects of Various Antibiotics and Vitamins on Development of Renal Lesions and Mortality (Experiments with *Ad libitum* Feeding).

Supplement to choline-free diet	No. of animals	Avg wt gain,* g/d	Animals with renal lesions: Dead, %	Animals with renal lesions: Alive, %	Animals with normal kidneys, %
0	67	2.5	77	22	1
Chloromycetin .1%	13	2.5	85	15	0
Penicillin 2500 u†	13	2.7	69	31	0
Aureomycin .1%	13	2.8	31	69	0
" .5%	26	1.8	4	8	88
Saline .2 cc†	28	2.9	64	36	0
B_{12} 2 μg in .2 cc saline†	21	2.9	52	43	5
B_{12} 60 μg/kg	12	2.4	50	50	0
Vit. E × 10	13	2.5	92	0	8
Vitamins, H_2O-sol., × 4‡	12	2.5	83	17	0
Choline .3%	10	3	0	0	100

* Wt gain during pre-lesion period (first 4 days on diet).

† Given as daily intraper. inj. Other supplements incorporated in diet.

‡ All water-soluble vitamins of diet, not including choline or B_{12}.

ether or after resolution by petroleum ether. Feces from controls and aureomycin-treated animals were collected into alcohol during the second, third and fourth days on the diets, and total choline determinations were carried out on the material obtained by Soxhlet extraction with methanol or by wet extraction after homogenization.

Results. Effects on renal lesions. The results of experiments with *ad libitum* feeding are summarized in Table I. Almost all of the animals on the unsupplemented diet developed the characteristic large tense "hemorrhagic" kidneys of choline deficiency, beginning near the end of the fourth day, and most of them died during the experimental period. Chloromycetin at 0.1% level, and penicillin in large doses, did not appreciably alter the results. On the other hand, aureomycin at 0.1% level appeared to decrease mortality, and at 0.5% level it largely prevented renal lesions and death of the animals. The data in the lower portion of Table I show that renal injury and death on the choline-deficient diet were not substantially affected by increasing greatly the amounts of vit. B_{12}, vit. E, or the remaining water-soluble vitamins of the diet. These results presumably indicate that the protection afforded by aureomycin was not entirely a result of conserving or increasing the production of any of these vitamins. The addition of choline to the diet resulted in complete protection. It should be noted that daily food consumption and gain in weight were reduced in the animals receiving the higher level of aureomycin, and it is well known that a sufficient reduction in food consumption will prevent development of renal lesions on a choline-deficient diet. However, we have observed in previous experiments that the daily weight gain must be reduced below 1 g or prevented entirely in order to achieve the degree of protection afforded by the aureomycin. Experiments with controlled feedings were carried out to clarify this question further.

In Table II, the results of experiments in which each animal received 3.6 g of food per day are summarized. The average daily gain in weight of the animals in each group was very nearly the same. The limitation in food consumption caused some delay in development of the renal lesions in the control group, as well as a moderate reduction in the incidence and severity of the lesions, compared with the results of experiments with *ad libitum* feeding. Again, however, aureomycin afforded an impressive degree of protection. Chloromycetin, terramycin, and streptomycin all prevented death, perhaps by controlling secondary infections, but only streptomycin appeared to reduce the incidence of the renal lesions and this reduction was much less than that observed with aureomycin. All of these antibiotics caused considerable enlargement of the caecum; this effect was slightly less marked with aureomycin than with the others. Chloro-

TABLE II. Effects of Various Antibiotics and Vitamins on Development of Renal Lesions and Mortality (Experiments with Controlled Feeding).*

Supplement to choline-free diet†	No. of animals	Animals with renal lesions: Dead, %	Animals with renal lesions: Alive, %	Animals with normal kidneys, %
0	77	38	44	18
Chloromycetin .6%	13	0	100	0
Terramycin .5%	10	0	80	20
Streptomycin .5%	11	0	55	45
Aureomycin .5%	59	0	20‡	80
" (reppt.) § .6%	13	0	8	92
" (inact.) ‖ .6%	13	54	46	0
B_{12} 60 μg/kg	13	15	31	54
Vit. E × 10	13	8	84	8
Choline .3%	5	0	0	100

* Each animal received 3.6 g of food/day.

† Concentrations of supplements calculated on basis of free base in cases where salts were employed.

‡ One-half of animals classified here as having renal lesions had very mild lesions.

§ Reprecipitated 3 times from acid solution.

‖ Inactivated by heating in alkaline solution.

mycetin produced diarrhea in some animals.

Effects of purification and alteration of aureomycin. When crystalline aureomycin HCl was further purified by repeated reprecipitation from acid solution, there was no reduction in its protective effect. On the other hand, when it was modified by heating in alkaline solution to the point of destroying its antibacterial action(3), its protection against choline deficiency was abolished, and it no longer caused enlargement of the caecum. These observations suggested the possibility of a relationship between the protective effect of aureomycin and its effects on the microorganisms in the gut. Under the circumstances of limited food consumption, vit. B_{12} appeared capable of affording definite protection, but less than that afforded by aureomycin.

Effects on other organs. The data in Table III, obtained from animals receiving 3.6 g of food per day, indicated that no change in size occurred in the thymus, adrenals, or thyroid glands as a result of administration of aureomycin. Similarly, no changes were observed on examination of microscopic sections of these organs. These studies made it appear unlikely that the protective action of aureomycin was due to effects on the thyroid or adrenals; the possibility of such a mechanism was suggested by our previous observation that thiouracil or cortisone afforded considerable protection against development of the renal lesions on a choline-deficient diet(4). In addition to producing the caecal enlargement already noted, aureomycin usually caused a reduction in the relative size of the liver (Table III).

Effects on liver lipid. The accumulation of lipid in the liver which occurred as a result of the choline-deficient diet appeared to be decreased slightly to moderately by aureomycin. The average value for total liver lipid in the untreated deficient groups was 9.5%, compared with 7.6% in the aureomycin-treated groups, and 5.0% in the groups receiving choline.

Effects on tissue choline. In Table IV are shown the results of an experiment on the effects of aureomycin on the choline concentration in liver and kidney tissue of animals on the deficient diet. It appeared from this and several other experiments that the tissue choline concentrations probably were increased to some extent by aureomycin.

Effects on fecal choline. Feces from aureomycin-treated animals in 4 experiments contained small but appreciable amounts of choline—0.3 to 1.5 mg per 2- or 3-day pool from 15 rats—while feces from animals on the unsupplemented choline-deficient diet contained no measurable quantities. Feces from animals receiving choline in the diet, like those from animals receiving aureomycin, were

TABLE III. Relative Wt of Organs.

	Diet (3.6 g/rat/day)			
	Choline-free†	Choline-free +.5% aureomycin		Choline-free +.3% choline
	4 days	4 days	9 days	9 days
Wt of organs (% of body wt)				
Liver	5.36	4.39	4.10	4.48
Kidneys	1.08	1.05	1.00	1.02
Heart	.50	.48	.40	.43
Thymus	.29	.25	.28	.29
Adrenals	.024	.026	.023	.023
Thyroid	.010	.010	.007	.007
Caecum*	1.30	4.08	5.34	1.34
Avg wt of animals, g	39.4	41.6	51.8	49.7

Each figure represents the avg of 5 determinations on individual animals. The data were quite homogeneous.

* Caeca were weighed without removing contents.

† Renal lesions were present in a few of animals on unsupplemented choline-deficient diet on 5th day, and in all of them on 9th day. The data in the table were obtained exclusively from animals which had not yet developed renal lesions.

TABLE IV. Choline in Tissues after 4 Days on Diets.

Diet (5 g/rat/day)	Avg daily wt gain per rat, g	Liver choline, mg/g wet wt	Kidney choline, mg/g wet wt
Part I			
Choline-free	2.8	1.54 1.25	2.13 2.06
" +aureomycin .5%	2.9	1.76 1.86	2.27 2.35
" +choline .3%	3.1	2.74 2.88	2.54 2.45
Part II			
Choline-free	2.5	1.63 1.67	2.02 2.07
" +aureomycin .5%	2.6	1.78 1.89	2.28 2.35
" +choline .3%	3.4	2.70 2.74	2.71 2.51

Results within each bracket represent determinations on duplicate pools of tissue from 5 rats. Determinations in this experiment were done on extracted material without petroleum ether resolution. Several animals on unsupplemented deficient diet had very early renal lesions without significant renal enlargement at the time of the determinations.

observed to contain small quantities of choline, but this might have occurred as a result of contamination of the feces with food. Aureomycin itself gave no reaction for choline when run through the procedures employed in the choline determinations on tissues and feces.

Discussion. Aureomycin has been observed to increase the growth of animals on presumably adequate diets(5), on diets deficient in certain vitamin factors(6), and when administered together with folic acid antagonists(7). This antibiotic also has been noted to decrease dietary hepatic injury in rats(8), and to produce favorable responses in pernicious anemia(9) and fibrocystic disease of the pancreas(10). The flora of the gut is markedly altered by aureomycin(11). Most investigators, while acknowledging the possibility of other mechanisms, have been inclined to attribute these effects of aureomycin to changes in production or utilization of various biologically-active substances in the gut as a result of quantitative or qualitative alterations in the microbial flora.

Since vit. B_{12} appeared to have some protective action under the circumstances of the present experiments, a part of the effectiveness of aureomycin under these circumstances may have been a result of increased production or conservation of vit. B_{12}. Aureomycin has been shown to increase the vit. B_{12} activity of the feces(12), and to decrease mortality on B_{12}-deficient diets(13). The vit. B_{12} activity present in the crystalline aureomycin itself was far too low to produce significant effects, and the choline content was negligible. There is no information available as to whether aureomycin contains labile methyl groups utilizable in the biological formation of choline; it appears unlikely that its protective activity was based on this mechanism. Studies on liver and kidney choline, and on fecal choline, suggested that an increase in formation or a decrease in destruction of choline, either in the gut or in the tissues, occurred as a result of aureomycin therapy. There is some question, however, of the specificity of the choline method, and there is no assurance that the protective effect of aureomycin was actually a result of the changes in choline content of the tissues. No information was obtained concerning possible decreases in the formation of harmful factors in the gut, or direct effects on tissue metabolism, as a result of aureomycin administration.

Summary. The renal lesions and animal mortality caused by a purified diet deficient in choline were largely prevented by supplementing the diet with rather high levels of crystalline aureomycin. Fatty changes in the liver also appeared to be reduced to some extent. These effects of aureomycin were not due to changes in food consumption, and did not appear to involve the endocrine glands. The effects of aureomycin were not diminished by recrystallization, but destruction of the antibacterial properties by heating in alkaline solution also abolished the protective effects. Studies with diets containing greatly increased amounts of various vitamin factors produced no evidence that the effects of aureomycin might have been due to alterations in production or conservation of any of the factors present in the diet, with the exception of vit. B_{12} and choline itself. The levels of choline in liver and kidney tissue and in feces of deficient animals appeared to be increased slightly as a result of aureomycin administration.

Aureomycin HCl was kindly supplied by Lederle Laboratories, Inc., chloramphenicol (Chloromycetin) by Parke, Davis and Co., and terramycin HCl and streptomycin sulfate by Charles Pfizer and Co., Inc. Vit. B_{12} was furnished by Merck and Co., Inc.

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Presence of Complement in Serum of the Mouse. (19642)

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The results of previous investigations(1-3) have indicated that serum of the mouse is incapable of acting as a source of complement in a hemolytic antigen-antibody combination. The results obtained by Ritz(1) indicated that Endpiece was not present, while Brown (2) concluded that the second component was lacking. Rice and Crowson(3) found that the titers of C′2 and C′4 were less than 10 units per ml of serum. By using fewer sensitized sheep erythrocytes it has been possible to demonstrate complement activity in most mouse sera.

Materials and methods. The Swiss strain of albino mouse, on which all titrations were performed, was obtained from breeding stocks

TABLE I. Titer of Complement in Individual and Pooled Mouse Sera.

Source		Sex	Units complement/ml serum
Individual	1	♀	2.5
	2	♀	1
	3	♀	1
	4	♀	<1
	5	♀	1
	6	♀	<1
	7	♀	<1
	8	♂	< .5
	9	♂	1
	10	♂	1
	11	♂	.5
	12	♂	1
Pool of 7 mice		♂ ♂	1
7		♂ ♂	1
2		♀ ♀	1

maintained in this laboratory. Both male and female adult animals were included. Blood was secured either by intracardial puncture or by snipping the brachial artery and withdrawing the blood from the pouch formed by the skin and the body wall. The resulting clot was broken up with a small glass rod and the tube containing blood was centrifuged at 2500 r.p.m. for 15 minutes. The serum was removed and utilized immediately. In order to secure titrations below 10 units per ml of serum, only one tenth the usual numbers of sheep erythrocytes, sensitized with rabbit-anti-sheep-cell amboceptor, were used. Each tube, therefore, contained 0.2 ml of sensitized sheep erythrocytes (approximately 12,500,000 red cells), varying amounts of undiluted mouse serum, and enough saline to make a final volume of 0.8 ml. Magnesium was routinely incorporated into all saline solutions. The tubes were shaken, placed in an incubator and held for 1 hour at a temperature of 37°C. The greatest dilution showing 100% hemolysis was taken as the endpoint and titers were expressed in terms of units per ml of undiluted serum.

Results. In 8 of 12 mice of both sexes, complement activity was clearly demonstrated (Table I). The highest titer obtained was 2.5 units per ml of serum and in 4 mice there was no discernible activity. The titers of the 3 pooled sera were 1.0 unit per ml each. There was no difference in titer in relation to sex. Heating fresh mouse serum to 56°C for 30 minutes destroyed complementary activity. No natural sheep cell hemolysins could be demonstrated in any mouse serum.

Discussion. The presence or absence of complement activity in a given serum is dependent on the criterion used to define the two terms. The titrations performed by Brown (2) as well as those of Rice and Crowson(3) would measure no less than 10 units of complement per ml of serum. Ritz(1), and Brown (2) concluded that certain components were absent, while Rice and Crowson(3) indicated a deficiency by utilizing the term of "less than 10 units." The earlier investigations were made without the benefit of magnesium in the system. Further studies may reveal yet additional substances to enhance complement activity and thus add further evidence of hemolytic complement activity in sera of animals thought to be deficient. It is perhaps wise, therefore, to question the reported total absence of hemolytic complement in the serum of animals such as the sheep, cow and horse until additional studies utilizing a system which would record titers of lower magnitude than those previously used, have been carried out.

Summary. Using a reduced number of sensitized sheep erythrocytes, hemolytic complement activity in the serums of most mice has been demonstrated.

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Humoral Factors Involved in the Induction of Liver Regeneration in the Rat.*† (19643)

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In previous work the relationships between liver regeneration in rats, age and carcinogenesis with azodyes were investigated(1,2). This is a report on further investigations with the emphasis on the mechanism controlling liver regeneration.

In vitro experiments. In order to investigate the possibility of the existence of humoral factors involved in liver regeneration, blood sera from partially hepatectomized and control rats were compared with respect to their action: a) on the initial growth response of primary rat liver explants cultivated *in vitro*, and b) on the growth period of *in vitro* cultures of a strain of normal rat fibroblasts. In the first series 24 male rats, 6 to 10 months of age grouped in pairs, were used. The experimental procedure employed on each pair was as follows: A partial hepatectomy consisting of the removal of the two main lobes of the liver was performed on one of the partners and a sham operation consisting of a laparotomy and manipulation of the liver on the other. Since hepatectomy reduces food uptake, the diet of the control was reduced in order that his food uptake would not exceed that of the hepatectomized rat. Twenty-four hours after the operation the animals were bled and the sera obtained were tested within 48 hours. Liver from a male rat 3 weeks of age was used as test tissue. A small piece of the tissue was cut up in the fluids to be used as culture medium and explanted directly on the glass wall of roller tubes without the use of plasma or embryo extract. Six tubes containing 6 fragments each were set up for each experiment. In 3 tubes the medium consisted of 50% serum from the hepatectomized animal plus 20% human placental cord serum, and in the other three, 50% serum from the control animal plus 20% human placental cord serum. The diluent was balanced salt solution (Gey). The tubes were allowed to stand in the incubator for 45 minutes in a vertical position in order to obtain good adherence of the fragments to the glass and then incubated in the rotating drum. Observations were made every 24 hours for a period of 6 days without renewal of the medium. The growth activity was estimated using as a criterion the fibroblastic outgrowth which always precedes epithelial outgrowth in such primary explants. Four grades of activity were recognized and defined as follows: Grade 0 activity was characterized by migration of macrophages and a few isolated fibroblasts without the formation of a network; Grade 1+ activity by the formation of a loose fibroblastic network involving only a limited area of the periphery of the fragment; Grade 2+ activity by the formation of a loose fibroblastic network involving a larger area of the periphery of the fragment; and Grade 3+ activity by the formation of a denser fibroblastic network involving most of the periphery of the fragment. Migration of liver cells accompanied the fibroblasts, but was not consistent enough to allow classification. Each group of cultures was graded on the basis of maximum activity found within the group.

The results are summarized in Fig. 1. In 11 out of 12 cases the activity of the cultures containing serum from the hepatectomized animals was greater than in the controls. In 7 cases the difference was striking since the controls showed no activity. In only one case was the activity in the control above Grade 1.

In the second series the action of sera from partially hepatectomized and control rats on

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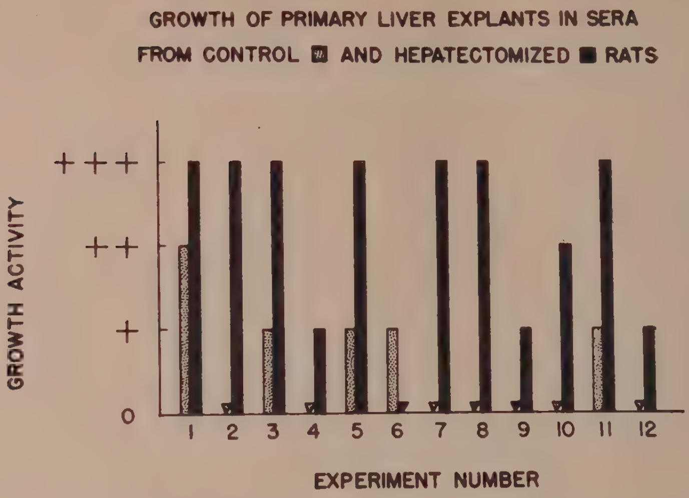
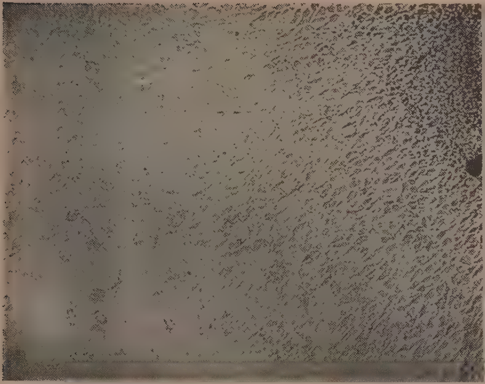


FIG. 1. Degrees of growth activity defined in text. The small triangles indicate 0 activity.

the growth period *in vitro* of cultures of a normal rat fibroblastic strain, *14pf*, was investigated. This strain has been isolated and grown *in vitro* in this laboratory since 1938 (3). The growth period is defined here as the interval between the beginning of cellular outgrowth of a newly transferred culture and the cessation of growth with degeneration under conditions which provide no renewal of the medium. The degenerative process characterizing the end of the growth period is such that no morphologically recognizable living cells can be seen within the growth zone of the colony (Fig. 2,3). Twenty-eight rats were used. The experimental procedure was essentially the same as in the first series except that no human placental cord serum was used. In each experiment 4 roller tubes of strain 14pf were set up with only the serum from the hepatectomized animal and 4 tubes with serum from the control. In each group of 4 tubes 2 contained 20% serum and the other two 50%. Thus the experimental and control sera were each represented in 2 concentrations. There was no renewal of the medium and observations were carried out every 24 hours as before. The results are summarized in Fig. 4. In the large majority of cases the growth period was longer in the experimental than in the control serum. In all cases the growth period was longer in the 20% than in the 50% serum concentration. Analysis of variance was the statistical procedure employed to evaluate the significance of these results(4). The difference between control and experimental sera was significant with P less than 0.01. The difference between the 2 serum concentrations was also significant with P less than 0.001.

Comment. These data indicate that rat serum under certain conditions exhibits an inhibitory effect on the outgrowth of tissue cells *in vitro*, since a decrease of the serum concentration in the medium resulted in a longer growth period. A longer growth period was also obtained with sera from partially hepatectomized rats. If these 2 findings are related the effect of the serum from partially hepatectomized rats could be mediated through the decrease of certain constituents of the serum following partial hepatectomy. These constituents at the concentrations normally present in the blood would be considered to exert a growth-inhibitory effect. It is known that partial hepatectomy induces marked alterations in the composition of blood serum as a result of the decreased amount of functioning hepatic tissue. Serum proteins, for example, are known to undergo a decrease up to 20% during the 24 hours following partial hepatectomy in the rat(5). In such a case should the decrease of serum constituents



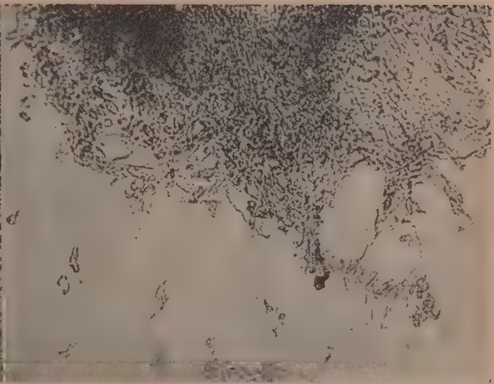


FIG. 2. 24-hr-old culture of strain 14 pf in 50% normal rat serum. Beginning of growth period. Growth zone composed entirely of living cells.

FIG. 3. 6-day-old culture of strain 14 pf in 50% normal rat serum. End of growth period. No morphologically recognizable cells within the growth zone.

be involved in the initiation of the regenerative process in the liver then a dilution of the serum *in vivo* through plasmapheresis might be expected to initiate mitosis in the intact liver of a rat.

In vivo experiments. Ten adult male rats, 10 to 12 months of age grouped in pairs, were used in a preliminary study and the experimental procedure employed on each pair was as follows: Under ether anesthesia and with aseptic conditions the left femoral vein of one of the partners was exposed and 5.0 ml of blood withdrawn through a silicone-treated syringe and needle containing 0.2 ml of 0.3% heparin solution. The skin wound was then washed with a 1000 u/ml solution of penicillin

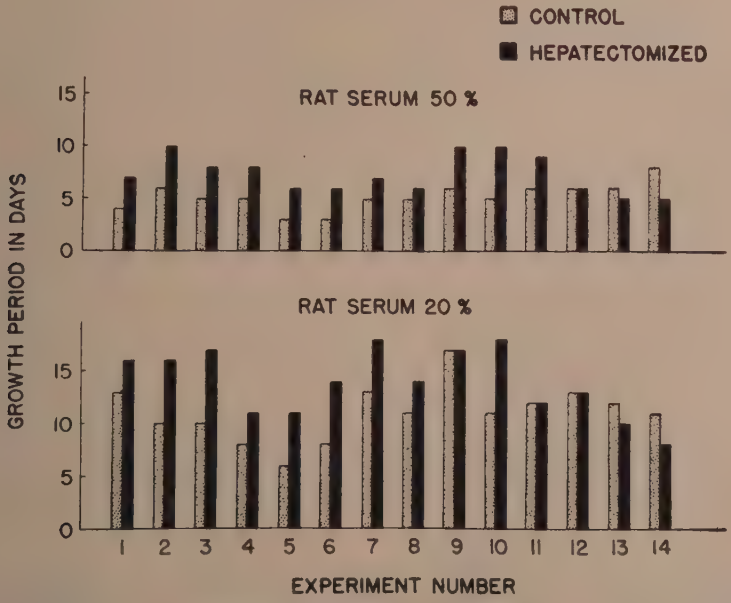


FIG. 4. Growth period of connective tissue strain 14 pf in sera from partially hepatectomized and control rats.

and closed with the aid of Michel clips. The animal was returned to his cage and allowed food and water *ad libitum.* The same procedure was repeated on the other partner of the pair but the blood was only withdrawn and reinjected immediately. The blood from the first animal was centrifuged, the plasma decanted, and its specific gravity measured by the copper sulfate method(6). The red cells were then washed twice in balanced salt solution and finally resuspended to a volume of 4.0 ml. Twelve hours later the right femoral vein was exposed under the same conditions as before and 7 ml of blood withdrawn from the experimental animal followed by reinjection of the previously prepared red cell suspension. The same procedure was used on the control rat but with immediate reinjection of the whole blood. The blood obtained from the experimental rat was centrifuged and again a red cell suspension made. The same procedure was then repeated (at 12 hour intervals from 24 to 84 hours), alternating the site of venipuncture. The amount of blood withdrawn at times was reduced to 5 ml and no reinjection of red cells was given. The animals were then sacrificed by exsanguination 12 hours after the last bleeding, a final determination of the specific gravity of the blood was made, and the livers removed and fixed for histological examination.

It was found that at the end of the plasmapheresis the specific gravity of the plasma of the experimental animals fell below 1.015. That of the controls remained above 1.019. Microscopic examination of the sections of the livers of the experimental animals showed a variable but marked increase in cell and nuclear size, an increased basophilia mostly in the juxtanuclear area of the cytoplasm, and numerous mitotic figures in all stages. Several fields containing no more than 100 nuclei exhibited 2 or even 3 mitotic figures (Fig. 5). It is known that in normal adult liver the ratio of dividing to resting nuclei is no greater than 1 to 10,000(7). The overall histological picture of the livers of the experimental rats showed a striking resemblance to the picture seen in the regenerating liver at 48 hours after partial hepatectomy(8). The microscopic examination of the sections of the livers of the control animals showed some increased basophilia of the cytoplasm of liver cells. In all other respects the histological picture of these livers was similar to the normal. Further work is now in progress aimed at standardizing the procedure of plasmapheresis in order to analyze quantitatively the mitotic activity thus induced and to establish any relationships with the degree of decrease in specific gravity of the plasma, and the length of time this decrease is maintained.

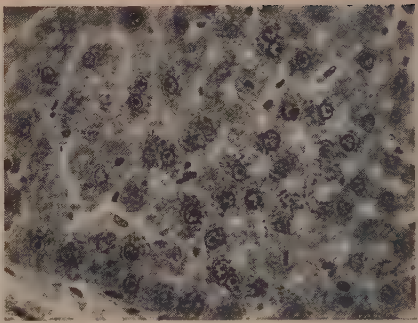


FIG. 5. Liver of an adult male rat submitted to plasmapheresis for 48 hr. Note large cells, variability of nuclear size, and deep basophilia of the cytoplasm. Three mitotic figures: one metaphase and two prophases. × 250.

Discussion. The growth response *in vitro* of primary liver explants and of a connective tissue strain was shown to be significantly greater in a medium containing serum from partially hepatectomized rats than in a medium containing normal adult blood serum from control animals. This indicates that following partial hepatectomy the composition of the blood serum is altered and that this alteration is possibly related to the initiation of liver regeneration. The results with varying concentrations of sera further indicated that certain constituents of blood serum exert a growth inhibitory effect *in vitro.* The decrease of the concentration of those constituents by dilution of the serum had an enhancing effect on the growth response of tissue cells *in vitro* similar to the one obtained with sera from partially hepatectomized rats. Likewise, by effecting a dilution *in vivo* of the blood serum of normal adult rats through plasmapheresis, a growth response in the intact liver similar to the one obtained by partial hepatectomy was induced. These last 2 findings suggest that the alteration in the composition of the blood serum following partial hepatectomy may in effect be the decrease of the concentration of certain constituents which at their normal concentrations exert a growth inhibitory action. Such a decrease, then, could account both for the enhancing effect of such serum on the growth response of tissue cells *in vitro* and the initiation of liver cell proliferation *in vivo* which follows partial hepatectomy. Further work is in progress in order to test in a direct way the validity of this hypothesis.

In their work on regeneration in parabiotic rats, Bucher, Scott, and Aub(9) suggested that chemical entities in the blood when present in sufficient quantities may cause the proliferative response in the liver. An elevated concentration of such substances was considered by them to be the stimulus initiating liver cell division following partial hepatectomy(9). The findings of the present work do not provide evidence in support of such a concept. On the other hand, their experimental findings concerning the induction of mitotic activity in the intact liver of one of the partners of a parabiotic pair by partial hepatectomy performed on the other are in complete agreement with the findings of the present work and can readily be interpreted on the basis of the hypothesis previously discussed.

Summary. On the basis of a comparison of the action of blood sera from partially hepatectomized and control rats on the growth response of tissue cells *in vitro* an hypothesis was formulated with regard to the induction of the regenerative process in the liver which follows partial hepatectomy. According to this hypothesis certain constituents of normal blood serum exert a growth inhibitory action at their normal concentrations. Partial hepatectomy reduces the amount of functioning hepatic tissue thereby resulting in a decrease of the serum concentration of these constituents. This in turn results in the initiation of regenerative growth in the liver. Further evidence supporting this hypothesis was obtained by the induction of cell division in the intact liver of adult rats by decreasing the concentration of serum constituents *in vivo* through plasmapheresis.

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Pricking Pain Threshold in Different Body Areas.* (19644)

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Although pain threshold measurements are customarily made using the forehead as the test surface, it is often desirable to use other body areas for this purpose. Measurements of cutaneous hyperalgesia or hypalgesia in a particular locality should properly include control measurements on the opposite side or have reference to measurements made on

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* These studies were aided by a contract between the Office of Naval Research and Cornell University.

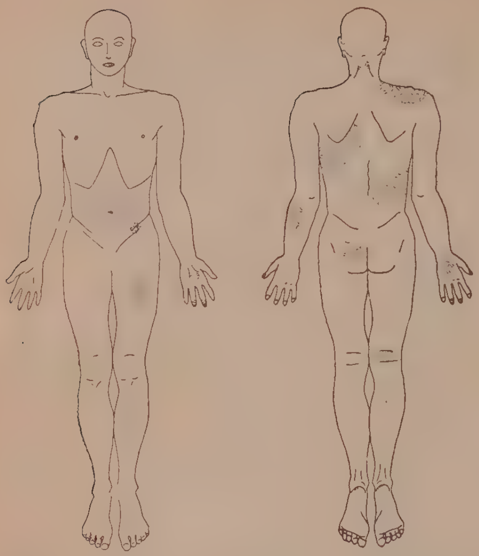

FIG. 1. Survey of sensitivity of various body regions. Stippled areas indicate regions of body surface on which pain threshold measurements were made.

normal subjects. Lack of definite information on this point has handicapped investigations in this laboratory(1,2). Whether the skin varies appreciably in its sensitivity to noxious stimulation in different body areas was also of sufficient interest in itself to warrant an investigation of the matter.

Method. The technic for measuring pain threshold by thermal radiation was used to make a survey of pain threshold level over the body surface. The skin surface to be tested was coated with India ink and the intensity of radiation just evoking a report of pain after a 3-second exposure was taken as the pain threshold. Just prior to each test the skin temperature was measured with a radiometer. Seven healthy subjects were used in these tests, 5 male and 2 female, all of whom were experienced in the recognition of the pricking pain threshold. The areas of the body upon which threshold measurements were made are shown in Fig. 1. Three test sites within each area were used and 3 measurements were made on each site. Room temperature during these tests was 20-25°C, and areas to be tested were exposed only during the time required to obtain a measurement.

Results. The results of the survey are shown in Table I, in which are given the average of the 63 values obtained for each body area, together with the standard deviations from the mean. The values given in the table have been corrected for temperature difference by the formula(3) $Q_{34} = Q + 20(T_s - 34)$ in which Q_{34} = pain threshold for a skin temp. of 34°C, Q = measured pain threshold, T_s = temperature of the area of skin being tested. It has been shown that correction for variations in skin temperature is necessary.

Also given in the table are the average values of skin temperature attained at the end of the 3-second exposure to the average threshold stimulus for each area. This temperature can be computed from a knowledge of the initial skin temperature, measured prior to ex-

TABLE I. Pricking Pain Threshold Measurements on Various Parts of the Body.

Skin area	Final skin temp., °C	Avg pain threshold, mc/sec/cm²
Forehead	45.7 ± 1.3	235 ± 27
Cheek (zygoma)	44.6 ± 1.3	210 ± 27
Nose	44.8 ± 1.6	215 ± 32
Lips	44.1 ± 1.9	200 ± 38
Chin	45 ± 1.3	220 ± 27
Chest	44.8 ± 2.2	215 ± 44
Nipple	43.8 ± 1.6	200 ± 32
Abdomen	43.9 ± .9	200 ± 18
Groin	43.3 ± 1.2	185 ± 24
Thigh (post.)	42.8 ± 1.4	175 ± 28
Thigh (ant.)	42.6 ± 1.4	170 ± 28
Knee cap	42.7 ± .9	175 ± 18
Popliteal space	43.5 ± 1	190 ± 20
Calf	43 ± 1.4	185 ± 28
Shin	43.9 ± 1.1	190 ± 22
Ankle	44 ± 1.1	190 ± 22
Dorsum of foot	43.2 ± 1.4	180 ± 28
toes	43.4 ± 1.7	185 ± 34
Arch	44.3 ± 1.4	200 ± 28
Heel	53.7 ± 2.2	390 ± 44
Heel, corneal layer shaved off		250
Shoulder	44.8 ± 1.8	215 ± 36
Nape of neck	43.8 ± 1.2	195 ± 24
Upper back	43.5 ± 1.6	185 ± 32
Middle	43.8 ± 1.2	195 ± 24
Lower	42.2 ± 1.4	160 ± 28
Buttock	42.4 ± 1.9	165 ± 38
Upper arm	43.4 ± 1.7	185 ± 34
Forearm, volar surface	44.4 ± 1.6	210 ± 32
outer	44 ± 1.2	200 ± 24
Back of hand	43.8 ± 1.6	195 ± 32
Finger pad	47.1 ± 2	260 ± 40
nail		215 ± 15
Palm (center)	45.1 ± 1.7	220 ± 34
over callus	45.6 ± 1.8	230 ± 36

posure, and the magnitude of the threshold stimulus(4). It has been pointed out that this value is more pertinent to the pain threshold, as determined by thermal stimuli, than radiation intensity or other measured quantity.

The values for male and female subjects have been included in the average as there was no significant difference in the measurements.

Comment. The pain threshold is approximately uniform over the body surface, as measured by the thermal radiation method. However, the heel of the foot has a significantly higher threshold due to the thick corneal layer of skin, but on the palm of the hand over a callus no significant elevation was observed. The finger pad threshold, on the other hand, exceeded the average for the body as a whole. Thresholds on the lower back, buttocks, and anterior and posterior aspects of the thighs were significantly lower than those on the forehead. This may be due to a thinner layer of stratum corneum overlying the pain endings in the back, although definite information on this point is lacking.

Summary. Using the thermal radiation method measurements on 7 subjects, the highest pain threshold values (390mc/sec/cm^2) were obtained on the heel of the foot. The lowest values of pain threshold (166-170 mc/sec/cm^2) were found on the lower back regions, buttocks, and thighs. Pain thresholds on all other body areas were approximately the same as that on the forehead.

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Localizing Properties of Anti-Rat-Aorta Antibodies.*† (19645)

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In order to investigate the localizing properties of antibodies prepared against a blood vessel which is not contained in any specific organ, we have studied antibodies prepared against rat aorta and are reporting the results here. Our interest in the *in vivo* localization of anti-blood-vessel sera stems from the evidence we presented that the localization of anti-kidney, -lung, and -liver antibodies in the liver, kidney and lung, as demonstrated by the use of radiolabeled antisera, takes place on the vascular bed of the organ(1,2). Antigenic differences have been found in the liver, kidney and lung with respect to their ability to localize antibodies, since the *in vivo* purification of anti-tissue sera yields antibodies of increased specificity for individual organs(1,2). Superimposed on these differences are antigenic similarities, because antibodies prepared against any one organ can localize in the others. Our present communications show that aorta also gives rise to antibodies capable of localizing in the liver, kidneys and lungs.

Experimental. Anti-rat-aorta sera were prepared as follows: aortas were excised from Sherman albino rats, scraped free of adherent tissue, washed and homogenized. Two rabbits received eleven injections each of 0.5 to 2 ml of a 10% aorta suspension on alternate days and were bled one week after the last injection. The sera were anti-RA452 and anti-RA457. Normal serum was obtained (NS 457) from rabbit 457 before immunization. The separation of the globulin fraction of the serum, the iodination thereof, the assay procedure and the details of the *in vivo* puri-

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† Part of this work was presented at annual meeting, Soc. Am. Bacteriol., Chicago, Ill., 1951.

TABLE I. Localization of Radioactivity in Various Tissues from I^{131} G-anti-RA. Rats were inj. with ½ ml solution containing about 1 mg protein.

Material inj.	Radioactivity inj., cpm × 10^{-6}	Rat wt, g	Radioactivity in tissue[*]								
			%/g/100 g rat					%/whole organ			
			Blood	Liver	Kidney	Lung	Aorta	Blood[†]	Liver	Kidney	Lung
I^{131} GNS-457	1.8	167	1.40	.16	.13	.23	.28	8.8	.61	.12	.11
		167	1.36	.10	.07	.21	.17	8.6	.47	.07	.11
		132	1.52	.11	.07	.37	.23	9.6	.53	.07	.16
I^{131} G-anti-RA457	1.2	161	1.84	.19	.19	.53	.45	11.6	.79	.18	.34
		177	2	.19	.24	.55	.38	12.6	1.20	.22	.29
		163	1.9	.18	.20	.50	.49	12	.74	.18	.34
I^{131} G-anti-RA452	1.7	160	1.50	.33	.24	.43	.29	9.5	2.2	.26	.21
		131	1.52	.25	.19	.43	.32	9.5	1	.24	.23
		165	1.80	.20	.24	.41	.40	11.3	.92	.20	.19

[*] Values are averages of duplicate samples.

[†] Calculated on basis of a blood volume of 6.3% (6).

fication method were described previously (1-5).[‡]

Results. The localization of anti-rat-aorta antibodies. The localization of radioactivity in the rats injected with I^{131} G-anti-RA and I^{131} GNS[§] and sacrificed 3 days later is shown in Table I. The results are given on a concentration basis as the percent of radioactivity, injected per 100 g rat, which localized per gram of wet perfused tissue; and on a total basis as the amount localized per whole organ.

It can be seen that there is an increased localization of radioactivity in the liver, kidneys and lungs of the animals receiving the two different anti-aorta sera as compared with the localization in those receiving the normal serum preparations. The increased localization of the antisera prepared against aorta is therefore similar to that of antisera prepared against the other tissues we have studied, namely, liver, kidney and lung (2,5,7). However, the two anti-aorta sera differ between themselves indicating differences in the antibody responses of the two rabbits toward the various antigens present in the injected tissue.

Thus, on a whole organ basis, serum 457 has less liver and kidney localizing activities but more lung localizing activity than serum 452. Such individual responses of different rabbits were also observed during the study of antisera formed against rat-liver-blood-vessel preparations (2).

Even though it appears that there is some localization in aorta from the anti-aorta preparations, we do not feel that these data are conclusive on this point.

Localization of in vivo purified anti-rat-aorta antibodies. Anti-rat-aorta antibodies were purified *in vivo* by the following procedure: rats were injected with I^{131} G-anti-RA, and were sacrificed and perfused 3 days later. The liver, kidneys and lungs were removed, homogenized and the insoluble sediments washed with saline. The *in vivo* localized antibody was eluted from the saline suspended sediments by adding sodium hydroxide to pH 10-11. The supernate was neutralized and injected into rats. The results are shown in Table II. The localization pattern of the *in vivo* purified material was determined by the organ of initial localization. Thus, the material eluted from liver localized to a greater extent in liver than that eluted from kidney or lung; the material eluted from kidney localized to a greater extent in the kidney than that eluted from the liver and lung; and the material eluted from the lung localized to the greatest extent in the lung.

Even with these increased specificities, cross reactions persist as follows: a) Liver eluted material localized to the same concentration in kidney as in liver.[‖] b) Kidney eluted material localized in liver but to a much lower concentration than in kidney. c)

[‡] Carrier free radioiodine (I^{131}) was obtained from the U. S. Atomic Energy Commission, Oak Ridge Operations, Isotopes Division, Oak Ridge, Tenn.

[§] I^{131}G-anti-RA, and I^{131}GNS refer to the radioiodinated globulin fractions of anti-rat-aorta serum and normal serum respectively.

[‖] A larger total amount localizes in liver than in kidney due to the larger size of the liver.

TABLE II. Localization of *In vivo* Purified Anti-RA. Rats were inj. with 1 ml of solution.

			Radioactivity in tissue*							
			%/g/100 g rat				%/whole organ			
Material inj.‡	Radioactivity inj., cpm × 10^{-3}	Rat wt, g	Blood	Liver	Kidney	Lung	Blood†	Liver	Kidney	Lung
G-anti-RA457										
ALi	9.35	103	1.51	2.6	2.7	.76	9.5	13.9	2.8	.63
AK	12.1	104	.74	[1.2]	17	1.4	4.7	[6.1]	15.6	.91
		84	.62	1.7	11	1.6	3.9	10	12.3	.98
ALu	2.6	87	(1.7)	(.6)	(1.1)	2.4		(3)	(1.2)	1.6
		91	(2.3)	(.7)	(1.3)	2.4		(4.2)	(1.6)	1.8
G-anti-RA452										
ALi	9.7	106		2.5	2.4	.8		13.8	2.1	.43
		96		2.7	2.3	(.4)		13.9	2.1	(.3)
AK	8.5	94		1.6	11.7	(.6)		7.4	12.9	(.4)
		87		1.8	12.2	(.8)		7.5	10.8	(.5)
ALu	5.2	110		(1.1)	2	3.9		(6.1)	1.9	2.76
Normal serum controls, avg of values of Table III, ref. 2										
NSLi				1.5	1.7			7	1.7	
NSK				1.2	5.1	2.3		6.4	5.2	1.4
NSLu					1	1.6				1.02

* Values are averages of duplicate samples except for those in brackets which are for one sample only. Values in parentheses are based on counts of 25/min or less above a background of 25/min and are not considered accurate.

† Calculated on the basis of a blood volume of 6.3% (6).

‡ The eluates are abbreviated to designate the antiserum originally inj. as the first symbol, and the organ from which elution was made as the second symbol. Thus, ALu is the eluate from the lung of an animal receiving anti-rat-aorta.

Lung eluted material localized in the kidney and liver but to a lower concentration than in lung. These results are similar to those reported previously for *in vivo* purified antibodies from other anti-tissue sera(1,2).

Discussion. Localizing antibodies are formed when rat aorta is injected into rabbits. When these antibodies are injected into rats, they localize in the liver, kidneys and lungs, demonstrating the presence in aorta of substances which can elicit the formation of antibodies capable of localizing in the kidney, liver and lung. The antibodies fixed in a particular organ are different from those localizing in other organs in that the localization patterns of *in vivo* purified antibodies isolated from various organs differ from each other.

Summary. Antisera prepared in rabbits against rat aorta contain antibodies capable of localizing in the liver, kidney and lung of the rat when injected intravenously. The antisera can be fractionated by *in vivo* purification to yield antibodies capable of localizing preferentially in liver, kidney or lung. This is interpreted as showing that the aorta has antigens in common with the vascular beds of these organs.

We wish to thank Rose Lipari, Ruben Medina, and Richard Clarke for their assistance in these experiments.

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Antidiuretic Effects of Physostigmine and Atropine in the Dog.* (19646)

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Intravenously administered acetylcholine can inhibit water diuresis in the dog, presumably by stimulating the hypothalamo-hypophyseal system to liberate antidiuretic hormone(1). The injection of acetylcholine or physostigmine directly into the region of the supraoptic nuclei of the dog has been shown to produce antidiuresis(2). Atropine, administered subcutaneously, has been employed in conjunction with intravenously injected acetylcholine to eliminate the muscarinic effects of the latter, and physostigmine, administered subcutaneously, has been used to "reinforce" the antidiuretic action of acetylcholine(1-4). It has been reported that large doses of atropine are antidiuretic in mice(5). In dogs this drug is said to inhibit an established water diuresis, or to delay and diminish diuresis(6,7). Ginsberg and Heller are quoted by Heller(8) as being unable to inhibit urine flow in rats with doses as high as 50 mg per animal administered subcutaneously.

During experiments in this laboratory on the antidiuretic effect of acetylcholine, atropine has been routinely used to block muscarinic effects, in doses of 0.05 to 0.15 mg per kg administered subcutaneously at the time of water loading. As in Pickford's work, no difficulty was encountered in achieving a satisfactory water diuresis. It was decided, nevertheless, to investigate the alleged antidiuretic activity of atropine. During attempts to potentiate the antidiuretic activity of 3-hydroxy-2-phenylcinchoninic acid, intravenously injected physostigmine was found capable of inhibiting water diuresis. The latter drug was therefore included in our antidiuretic studies. The data reported in this paper are the results of these investigations.

Methods. Trained perineotomized female dogs were used for all experiments, urine being collected by catheter at 15-minute intervals. Atropine was injected as the hydrochloride and physostigmine as the sulfate. All doses are calculated as these salts. Each injection was administered intravenously in a volume of 5 ml or less over a period of 30 seconds or less. Doses were usually varied by 2-fold increments. Injections were made after diuresis was established by the administration of 40 ml of water per kg by stomach tube. An antidiuretic effect was considered obtained when an unequivocal decrease in urine flow was observed, followed by a return to higher levels, as described previously(9). In the majority of experiments, the urinary excretion of endogenous creatinine was used to detect changes in glomerular filtration rate, but in 4 experiments the clearance of administered creatinine was used. The method of Folin-Wu as modified by Phillips(10) was employed to determine creatinine.

Results. Ten experiments with physostigmine were performed in 4 dogs. Doses of 0.08 to 0.1 mg per kg were necessary to produce an antidiuresis in all animals. The changes in urine flow were independent of changes in glomerular filtration rate (Fig. 1) and therefore the antidiuretic effect may be

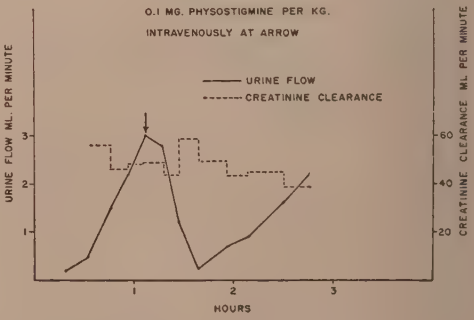

FIG. 1. Effect of physostigmine on water diuresis and creatinine clearance in the dog.

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† Sharp and Dohme Fellow in Pharmacology and Experimental Therapeutics.

said to represent increased tubular reabsorption of water. Urine flow in almost all instances decreased within 15 minutes after injection of the drug, with the maximal inhibition occurring at approximately 30 minutes. A slow return to pre-injection urine flows followed. Doses of 0.04 to 0.12 mg per kg produced in all dogs tremor of varying severity lasting from 8 to 50 minutes. In one dog, profuse salivation and defecation also occurred.

Eighteen experiments with atropine were performed in 5 dogs. In 3 dogs unequivocal antidiuretic effects were observed with doses of 0.12, 0.25 and 0.5 mg per kg, respectively. As with eserine, the changes in urine flow were independent of changes in glomerular filtration rate. In most instances where an antidiuretic effect was elicited the onset of the effect was delayed 15 to 45 minutes after injection of the drug, and the maximal effect was reached 30 to 60 minutes after injection. Urine flows returned slowly to pre-injection values. In the other 2 dogs no inhibition of urine flow could be detected despite the administration of doses (0.25 to 0.5 mg per kg) sufficient to produce toxicity. Higher doses could not be administered to these particular animals because of the production of excitement or viciousness severe enough to make experiments impossible. Even minimal effective doses were accompanied by marked stimulation of the central nervous system in 2 dogs. This stimulation reached maximum intensity during the period of the antidiuresis. The toxic manifestations seen included restlessness, whining, barking, ataxia, panting, vomiting, mydriasis, and tachycardia.

Discussion. Either of the 2 drugs studied is capable of producing antidiuresis in dogs by increasing the renal tubular reabsorption of water, an effect most probably mediated by release of antidiuretic hormone from the neurohypophysis. The doses necessary to affect the urine flow, however, produce obvious signs of intoxication, although the production of toxic manifestations is not necessarily accompanied by antidiuresis. In the case of atropine, at least, it seems likely that the antidiuretic effect is part of the results of widespread stimulation of the central nervous system. In view of the large doses needed when the drug is given intravenously it seems unlikely that the relatively small doses used subcutaneously to block the muscarinic effects of acetylcholine could have significant effects on urine flow. The results with physostigmine are compatible with the theory of Pickford and associates that acetylcholine is the mediator of stimuli in the hypothalamo-hypophyseal system.

Summary. Physostigmine and atropine produce antidiuresis in the dog by increasing tubular reabsorption of water. The doses required, however, are large and usually accompanied by signs of toxicity.

We wish to thank Miss Sara Ann Verplanck for technical assistance.

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Nephrotoxic Action of Spermine. (19647)

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(Introduced by Floyd S. Daft.)
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Spermine is an aliphatic amine [NH_2-$(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$] that is widely distributed in mammalian tissues, with the highest concentration in the prostate gland (1,2). By the use of ion exchange resins we have developed a colorimetric method for the estimation of spermine, applicable to small amounts of material(3). As an illustration of the amounts present in the tissues of rats and mice, spleen contains from 300 to 600 μg per gram of wet tissue, and liver approximately half this quantity. Values within this range have been reported for man and large animals(1,4). Little is known of its physiological significance; it has been found to inhibit oxidation of carbohydrate in brain tissue *in vitro*(5); to be a growth factor for a strain of *Hemophilus parainfluenza*(6); to antagonize the bacteriostatic action of atabrine on *E. coli*(7); and to possess bacteriostatic action under certain conditions upon the tubercle bacillus(8). It has been generally considered, along with the related diamines putrescine and cadaverine, to be of low toxicity. The acute toxic effects of these amines have been attributed to a depressant action on the central nervous system(9).

It was observed that animals surviving the acute depressant action of spermine would often die after a lapse of several days. At autopsy marked kidney lesions were found, and further study has shown spermine to be a potent agent in the production of renal damage. Three commercial samples of spermine 4 HCl* bearing different lot numbers were used. One of them was recrystallized as the phosphate and another was put through one recrystallization as the hydrochloride and two as the phosphate. Satisfactory elementary analyses were obtained on these samples. A further sample of spermine phosphate was obtained through the courtesy of the National Institute for Medical Research of Great Britain. A sample of spermidine phosphate was obtained from Dr. Milton Silverman of this Institute, while putrescine and cadaverine (di HCl) were obtained from commercial sources. 0.5% solutions in 0.9% NaCl were employed. The phosphate was brought in solution by heating, and injections of this salt were made before the solution cooled sufficiently to crystallize out. All dosage values are expressed in terms of the base.

It is observed (Table I) that intraperitoneal injections into mice of 60 mg/kg or 2 daily injections of 30 mg/kg were uniformly fatal within 6 days to both male and female animals. One dose of 30 mg/kg killed 9 of 12 females. Eight of 10 mice receiving daily amounts of 15 mg/kg died after the 5th to 7th injection. Nine daily injections of 7.5 mg/kg were non-fatal.

Five rats received one injection of 33 mg/kg intraperitoneally. Animals sacrificed on the 3rd to 7th day showed moderate to advanced renal lesions.

Two small rabbits (1200 g) received single intraperitoneal injections of 33 mg/kg. No symptoms were observed until the 5th day when death in coma occurred. Advanced renal lesions were present. The non-protein nitrogen in the blood of these animals, up to the time of death, is shown in Table II. Similar determinations upon mice sacrificed at various times after one injection of 60 mg/kg are also shown.

Urines from the rats and rabbits contained considerable amounts of protein and many samples reduced Fehling's solution. Single intravenous injections of 5 to 15 mg/kg into rabbits produced proteinuria for several days.

This nephrotoxic action was not observed following putrescine or cadaverine (Table I). Mice were administered 200 mg/kg daily for 5 days and sacrificed on the 7th day. No renal lesions or elevation of blood N.P.N. were observed. Experiments with spermidine were

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* From Hoffmann-La Roche, Nutley, N. J.

TABLE I. Toxicity of Spermine Salts for Mice. Females employed unless otherwise indicated.

Preparation	Daily dosage, mg/kilo*		Route	No. of mice	No. dead	Time of death
Spermine 4 HCl	30 mg	× 1	I.V.	4	4	Immediate
Phosphate†	60	1	I.P.	11§	11	1 in 2 hr, others 3-4 days
	30	1		10§	5	3-5 days
	15	1		10§	1	3 "
" ‡	60	1		6	6	1 in 2 hr, others 3-4 days
	30	1		6	4	3–6 days
	15	1		6	0	
HCl	60	2		10	10	2
	30	2		5	5	2–4
	15	7		10	10	5–8
	7.5	9		5	0	
Phosphate	60	1		6	6	4–6
	25	5		10	10	4–6
Spermidine phosphate	60	1		3	0	
	30	1		3	0	
	17	1		3	0	
Putrescine 2 HCl	200	5		5	1	4
Cadaverine 2 HCl	200	5		5	0	

* Expressed as base.

† Recrystallized several times from the hydrochloride by Dr. E. L. Jackson of this Laboratory.

‡ Obtained from the National Institute for Medical Research, Great Britain.

§ Includes 4 male mice.

TABLE II. Blood Non-Protein Nitrogen Values Following Intraperitoneal Injections of Amines. Mice and rats were sacrificed at stated intervals to obtain blood, while rabbits were bled from ear vein.

				Blood N.P.N., mg %				
				Days after onset of treatment				
Animals	Compound	Dosage, mg/kilo		0	1	2	3	4	5–7
Mice	Spermine phosphate	60	× 1	45	70	64	350	290–367	
Rabbit	" "	33	1	40	62	83	161	322	
"	" "	33	1	40	53	108	170	294	
Rats		33	1	43–44					80–70–50*
Mice	Putrescine	200	5						43–59
"	Cadaverine	200	5						43–54
"	Spermidine	60	1						57†

* Values obtained on the 5th, 6th, and 7th days, respectively.

† 6th day.

limited by lack of material, but no renal injury was observed.

The early (1 day) morphologic changes following the injection of spermine consisted of hyaline droplet degeneration and fatty change in the epithelial cells of the proximal convoluted tubules of the kidney. One and a half days and in some instances even one day following the injection there was a diffuse desquamation of the epithelial cells of the proximal convoluted tubules, apparently most marked in the distal portion (outer medullary zone). There were no reactive inflammatory changes associated with the tubular necrosis. The structures of the distal nephron appeared well preserved except for the presence of numerous casts within their lumina. These casts were for the most part of the hyaline type, being periodic acid-Schiff positive. On occasion amorphous as well as definitely granular basophilic material was noted in the casts (hematoxylin and eosin stained sections). This substance was also in instances periodic acid-Schiff positive as well as being positive when stained by the Feulgen and by the Van Kossa methods. These latter technics indicate the protein, mineral (phosphate, probably calcium phosphate) and nuclear debris content of the casts. The lesions are similar in appearance from 3 to 10 days following the injection of spermine although at 10 days large lakes of mineral deposits (probably calcium phosphate) are noted within the lumina of the tubules of the distal nephron.

In all sections studied no glomerular, vascular or stromal changes were evident and lesions in the kidney as described above were similar in sacrificed animals to those observed in animals dying after the injection of spermine.

Other tissues failed to reveal significant morphologic change except for the presence of rare foci of necrosis in the liver of several animals surviving for 4 days after the spermine injection.

Animals injected with spermidine, putrescine and cadaverine failed to reveal any morphologic changes in the viscera.

Conclusions. Spermine administered parenterally is highly active in producing renal injury in mice, rats, and rabbits. This property was not observed for spermidine, putrescine, or cadaverine.

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Studies on Nicotinamide Inhibitors. (19648)

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Pyrazinoic acid was reported to be active in the cure of human pellagra(1). This compound, however, was later found to have no nicotinic acid activity for increasing the V-factor of human blood(2). Other investigators reported pyrazinoic acid and, in some cases, the corresponding amide to be inactive for the cure of canine blacktongue(3), growth of isolated pea roots(4), and for the growth of various microorganisms, for example, *Leuconostoc*(5), *Pasteurella*(6), *B. dysenteriae*(6), and *Proteus vulgaris*(7). These compounds have not been reported as nicotinamide antagonists for microorganisms.

Of a series of pyrazine and pyridine derivatives which were assayed for antinicotinamide activity with *Lactobacillus arabinosus*, strain 17-5, pyrazinoic acid, pyrazinamide and 2 sulfanilamido-5-nitropyridine are shown to be reversible inhibitors of nicotinamide. Experiments with pyrazinamide on rats and chicks are also reported.

Experimental methods. The details of the microbiological assays and the animal tests are given in a previous publication(8). The compounds tested for inhibitory activity with *L. arabinosus* were initially assayed at levels of 0.5, 1.0, 3.0, and 5.0 mg per ml alone and in the presence of 0.02 μg per ml of nicotinamide. This level of nicotinamide alone gives approximately half-maximum growth. The compounds showing inhibition were retested at 7 levels from 0.5 to 15 mg per ml against 7 levels of nicotinamide ranging from 0.005 to 0.2 μg per ml. An inhibition index was then calculated, which represents the ratio of inhibitor to vitamin producing a 50% inhibition of growth. The compounds were ad-

TABLE I. Inhibition of Nicotinamide Response of *L. arabinosus*.

Compound	Nicotinamide conc., γ/ml	Inhibition index × 1000
Pyrazinamide	.005	200
	.010	200
	.020	250
Pyrazinoic acid	.005	100
	.010	100
	.020	150
2-sulfanilamido-5-nitropyridine	.005	250
	.010	200
	.020	250

TABLE II. Reversibility of Pyrazinamide Inhibition of Nicotinamide.

Pyrazinamide conc., γ/ml × 1000	Nicotinamide conc., γ/ml	ml of .1 N NaOH	
		A	B
	.005	2.24	2.26
1	.005	1.91	1.87
1	.010	2.40	2.36
2	.010	3.83	3.80
2	.010	2.00	2.01
	.020	4.15	4.13
5	.020	5.90	5.92
5	.020	2.92	2.87
	.040	7.98	7.90

TABLE III. Effect of Pyrazinamide on Nicotinamide Growth Response in Rats.

Treatment	Initial wt and No. ( )	Avg gain and No. alive ( )	
		2 wk	4 wk
Basal diet	40(9)	14(9)	18(7)
+50 γ Nicotinamide daily	40(9)	25(9)	53(9)
+100 γ " "	43(9)	36(4)	84(4)
+200 γ Pyrazinamide "	40(9)	15(9)	23(9)
+10 mg " "	40(9)	17(9)	30(9)
+10 mg Pyrazinamide +50 γ Nicotinamide	41(9)	21(9)	50(9)

ministered orally once each day to the rats and chicks in aqueous or 25% ethanol solutions at the levels indicated in the tables.

Results. The following 34 compounds were found to be without anti-nicotinamide activity: Pyridine-3,5-dicarboxylic acid, Pyrazine-2,3-dicarboxylic acid, 4-Methylpyridine-2,3-dicarboxylic acid, Pyrazine-2,3-dicarboxamide, 3-Bromopyridine, 2-Methyl-3-amino-4,5 bis (aminomethyl) pyridine • 3 HCl, N-Thiazolylpyrazinamide, N, N Dimethylpyrazinamide, N-Methylpyrazinamide, N-Pyrazinylthiourea, N-Methylolpyrazinamide, N-Diethyl pyraiznoylaspartate, N-Pyrazinoylpiperidine, N-Isobutylpyrazinamide, N-(2-pyridyl) pyrazinamide, N (3-pyridyl) pyrazinamide, N-Phenylpyrazinamide, N-Hexadecylpyrazinamide, 3-Pyrazinoylamidoquinoline, N-(β-hydroxyethyl)N′-pyrazinoylethylenediamine, 3-Hydroxypyridazine-6-carboxamide, 2-Pyrrolidone-5-carboxamide, 2-Chloroquinoxaline-3-carboxamide, 1-Thiazolylpyrrole-α-carboxamide, N-Thiazolylthiophene-α-carboxamide, Desoxypyridoxine HCl, Salicylamide, Furoic acid, Furanilide, Pyrazinohydrazide, 1-Carbethoxy-4(α,β-dicarbethoxyethyl) piperazine HCl, N-(p-Methoxybenzyl) pyrazinamide, Pyrazinohydroxamic acid, Ethyl N-pyrazinoyl-β-alanate. None of the compounds showed any growth stimulating activity for *L. arabinosus*.

Three compounds, pyrazinamide, pyrazinoic acid and 2-sulfanilamido-5-nitropyridine reversibly inhibited nicotinamide for this microorganism. The inhibition index for each compound was constant over a 4-fold range of concentration of the vitamin as shown in Table I. Extension of the range was not feasible because of the insolubility at higher concentrations of the compounds. Table II gives some typical data illustrating the reversibility of the inhibitions, using pyrazinamide as the example.

The effects of pyrazinamide on the response of rats and chicks to nicotinamide are shown in Tables III and IV. No clear-cut inhibition in these species by the use of this compound could be demonstrated. A slight, probably non-specific interference, in the response of the chick to nicotinamide was observed.

Discussion. The early work, previously mentioned, on the lack of nicotinamide activity of pyrazinamide and pyrazinoic acid for several species of bacteria and animals, has been confirmed and extended. It appears that these compounds, under the conditions described for *L. arabinosus*, are relatively weak inhibitors, which can be readily reversed by small amounts of nicotinamide. A number of closely related compounds were found to be inactive in this respect.

The animal tests would indicate that pyrazinamide is not an antagonist for rats or chicks. It was not found to be toxic for dogs at the levels used by Waisman *et al.*(3). It

TABLE IV. Effect of Pyrazinamide on Nicotinamide Growth Response in Chicks.

Level of nicotinamide, γ daily	Level of pyrazinamide, γ daily	Initial wt and No.	Avg gain and No. alive ( )	
			2 wk	4 wk
50		40 (27)	17 (22)	41 (14)
50		42 (10)	40 (10)	80 (9)
100		40 (28)	35 (25)	99 (21)
200		39 (37)	45 (37)	166 (30)
	200	40 (8)	4 (6)	— (0)
	1 mg	39 (9)	25 (8)	44 (6)
	10	40 (19)	19 (15)	35 (7)
	100	38 (10)	9 (8)	40 (7)
200	1	39 (9)	37 (8)	111 (7)
50	10	42 (10)	28 (10)	57 (9)
100	10	42 (10)	38 (8)	82 (8)
200	10	40 (19)	46 (18)	119 (17)
100	100	38 (10)	28 (9)	96 (9)
200	100	38 (10)	30 (10)	126 (10)

is improbable that large enough doses could be given to animals to induce pellagra, if its inhibition index for *L. arabinosus* is an indication of the amount required.

Summary. 1. Of a series of 37 compounds tested, pyrazinamide, pyrazinoic acid and 2-sulfanilamide-5-nitropyridine were found to be nicotinamide antagonists for *L. arabinosus.* 2. Pyrazinamide was not an antagonist for rats or chicks.

The authors are indebted to Dr. S. Kushner, H. Dalalian, and J. L. Sanjurjo for preparing most of these compounds.

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Changes in Glucose Tolerance as an Index of Improvement after Electroshock Therapy. (19649)

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Impairment of glucose tolerance in many patients with manic-depressive, involutional and schizophrenic psychoses is well known. An attempt was made to evaluate the possible use of changes in glucose tolerance after electroshock therapy as an objective index of clinical improvement.

Material and methods. Twelve patients, ranging in age from 32 to 73 years, were the subjects; 10 were women. One of the patients was known to have had diabetes mellitus for a decade; the disease was discovered here in 2 others. The patients were given 3 electroshock treatments weekly in the numbers indicated (Table I). All patients were much improved following treatment. Studies of the blood sugar were made after oral administration of 100 g of dextrose before the first and again 5 to 10 days after the last treatment; the method of Folin and Wu(1) was used.

TABLE I. Diagnoses of Patients and Treatments Given.

Case	Age	Sex	Treatment, shocks	Diagnosis
A	65	♀	5	Involutional psychosis; melancholia
B	44	♀	6	Schizophrenia, paranoid
C	66	♀	5	Manic depressive, manic
D	54	♀	10	Involutional psychosis; melancholia
E	72	♀	12	" " "
F	40	♀	13	Schizophrenia, paranoid
G	54	♀	9	Paranoid condition
H	32	♀	4	Manic depressive, depressed
I	56	♂	4	" " "
J	59	♀	7	Involutional psychosis; melancholia
K	73	♂	7	" " "
L	58	♂	5	Manic depressive, depressed

Observations. Insignificant changes in glucose tolerance occurred in 3 patients (Fig. 1, Cases A, B, I) following a course of treatments. Slight impairment of glucose tolerance was noted in 2 instances (Fig. 1, Cases C, G), and a notable lowering of the level of the curve occurred in 4 (Fig. 1, Cases D, E, F, H). Improvement in sugar tolerance occurred after shock therapy in 2 of the 3 diabetic patients; this was definite in one instance (Fig. 2, Case K) and questionable in the other (Fig. 2, Case L).

Discussion. The observations made here show no consistent change in glucose tolerance in patients who improved clinically after a course of electroshock therapy; while the glucose tolerance may improve somewhat in some diabetic and non-diabetic psychotic patients treated with electroshock therapy, this change was inconstant. It is evident even from the results in this small group of patients that changes in glucose tolerance cannot be used as an objective index of improvement after electroshock therapy.

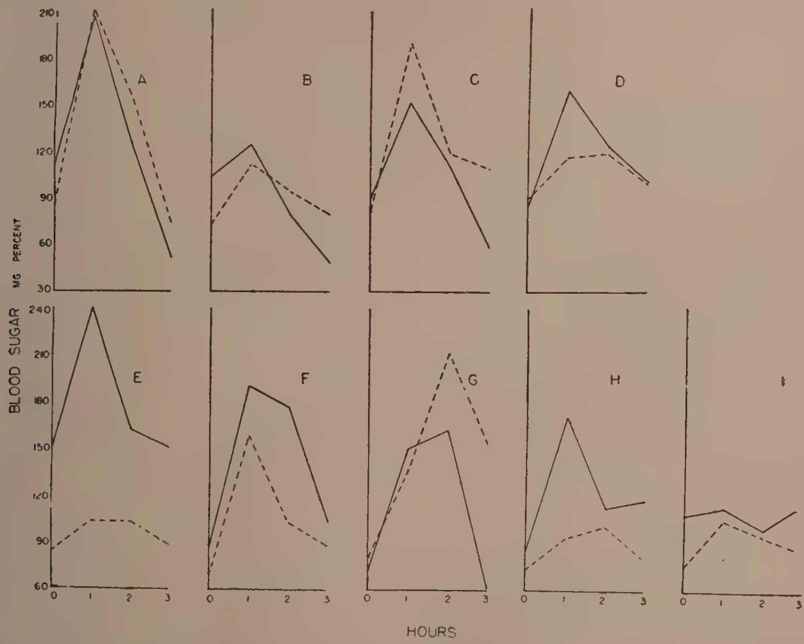

FIG. 1. Oral glucose tolerance tests before (solid line) and after (broken line) a course of electroshock therapy.

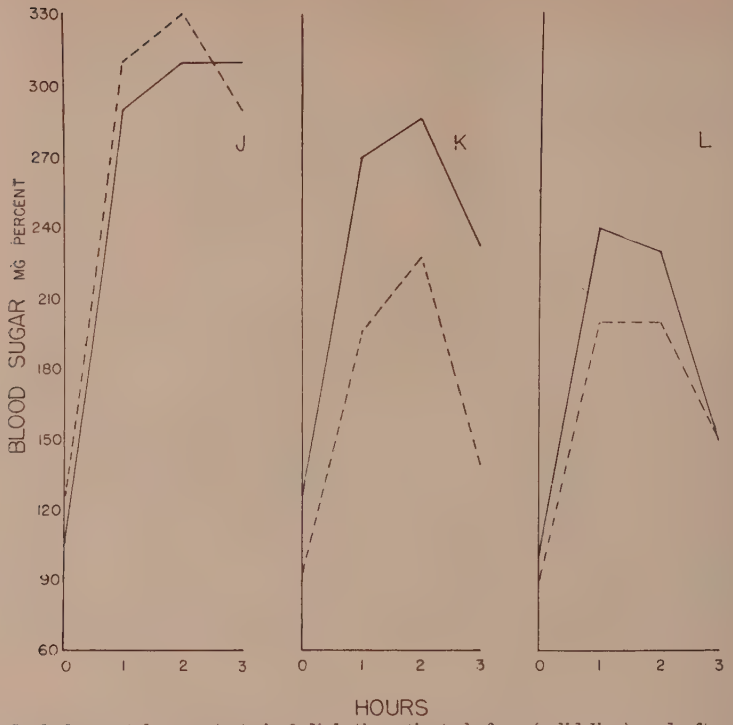

FIG. 2. Oral glucose tolerance tests in 3 diabetic patients before (solid line) and after (broken line) a course of electroshock therapy.

A number of factors may influence glucose tolerance in untreated patients with mental disease: these include metabolic changes associated with the mental illness itself, abnormal absorption from the gastrointestinal tract(2), and starvation. Hyperactivity of the adrenal cortex that occurs early in the course of electroshock therapy tends to depress glucose tolerance, but the hyperactivity induced by shock therapy wears off as treatment continues (3). However, the effect of adrenal cortical hormones on sugar tolerance may persist for weeks(4). In addition, the state of the sodium metabolism appears to have a profound influence on utilization of glucose when the adrenal cortex is stimulated(5); marked changes in sodium metabolism occur during the course of shock therapy(6,7). The effect of electroshock therapy on sugar metabolism is the resultant of the effects of changes in adrenal-cortical function, amelioration of mental disease, improvement in nutritional status, modification of electrolyte metabolism, and possibly other factors. These factors vary in different patients; the large number of irregularly variable factors involved makes correlation between changes in glucose tolerance and in clinical state a poor one.

Summary and conclusions. The effect of electroshock therapy on glucose tolerance was studied in 12 psychotic patients who improved with electroshock therapy. Changes in sugar tolerance varied in direction and degree; there was no correlation with clinical improvement.

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Significance of Intermedin Activity in Adrenocorticotropic Hormone Preparations.* (19650)

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Recent investigations by Johnsson and Högberg(1) and by Sulman(2,3) have suggested that intermedin and adrenocorticotropic hormone (ACTH) were closely related or identical, and that assay of melanophore expansion activity in the frog could be employed as a convenient test for ACTH. In addition, the physico-chemical properties(4) of the melanophore expanding hormone are in many respects similar to those reported for ACTH. The following experiments were designed to demonstrate whether the adrenal ascorbic acid depleting activity of various ACTH preparations is correlated directly with intermedin activity.

Assay methods. Hypophysectomized frogs, *Rana pipiens,* were employed for intermedin assay in most of these studies. In one series of experiments *Rana catesbiana* was employed. Quantitation of melanophore expansion was made on an arbitrary 1+ through 4+ scale, in addition to the determination of the minimal effective dose (m.e.d.) necessary to produce a 1+ reaction. Readings were made at half hour intervals for a 4 hour period after injection of the test solution (0.1 ml) into the dorsal lymph sac. Adrenocorticotropic hormone preparations were assayed by the adrenal ascorbic acid depletion test(5).

Results. The investigation proceeded along 5 separate lines. The first approach involved the determination of both types of activity in a number of ACTH preparations(6). The results of this series of experiments are presented in Table I. It is obvious that no direct correlation exists between the ACTH and intermedin activities of the various preparations. As a matter of fact, the few preparations tested show an inverse correlation.

TABLE I. Adrenocorticotropic and Intermedin Activities of ACTH Preparations.

Preparation, ACTH	ACTH activity, I.U./mg	Intermedin activity (m.e.d.), μg
L 2317 H	35	.5
M	30	.1
L 2316 C*	200	5

* Partial acid hydrolysate of L 2317 M(6).

The second series of experiments was undertaken in the hope of demonstrating a chemical separation of the adrenocorticotropic and intermedin activities. Fractions with highly potent adrenocorticotropic activity, obtained from a method using charcoal chromatography (7), exhibited a marked concentration of intermedin activity as well, and no separation was achieved. Definite separation was obtained, however, with fractions isolated from cellulose columns by a discontinuous pH gradient. As has been reported previously(8), almost all of the adrenocorticotropic activity resides in the second of three peaks obtained by this method. Intermedin activity, on the other hand, was almost completely concentrated in the first peak (Table II). Thus, an effective

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TABLE II. Distribution of Adrenocorticotropic and Intermedin Activities in the Fractions Obtained by Employing a Discontinuous pH Gradient on Cellulose Columns.

Peak*	Exp.	ACTH activity, I.U./mg	Intermedin activity (m.e.d.), μg
1	a	<4	< .5
	b	3	< .5
	c	6	< .5
	d	<4	<1
	e†	3	<1
	f	<3	<<1
	g	4	.1
2	a	40	>5
	b	120	5
	c	180	5
	d†	130	>1
	e	60	>1
3	a	<4	10
	b	<4	>2
	c	4	>2
	d	5	>2
	e	4	5
	f†	10	>1
	g	8	>1

* See reference (8).

† These solutions were assayed in *Rana catesbiana*.

TABLE III. Migration of Adrenocorticotropic and Intermedin Activities During Zone Electrophoresis on Paper.*

Fraction	ACTH activity, I.U./mg	Intermedin activity†
Anode	<7	3+
Origin	30	2+
Cathode I	70	1+
II	<7	±

* Borate buffer, pH 8.5, was used; a potential of 6 volts/cm paper was applied 19 hr, at 5°C.

† All solutions were assayed at 1 μg level. An arbitrary 1+ - 4+ scale of increasing intensity of darkening was employed for grading assay response.

TABLE IV. Effect of Alkali-Heat Treatment on the Adrenocorticotropic and Intermedin Activities of an ACTH Preparation.

Preparation	Exp. No.	ACTH activity, I.U./mg	Intermedin activity (m.e.d.), μg
Control	1	30	>1
	2	30	2
Alkali-heat treatment	1	1.5	<1
	2	1.4	< .5

separation of the bulk of the two activities has occurred.

Partial separation of the two activities was also obtained by employing zone electrophoresis on paper(9). For the experimental conditions employed (pH 8.0-9.0), the bulk of the ascorbic acid activity is to be found at the origin and in its neighboring cathode areas(6). When solutions obtained by extracting various sections of such papers were assayed for melanophore expanding activity (Table III), the region of greatest activity was found in the anode fraction where not more than a small percentage of the total ACTH activity could be demonstrated; whereas the cathode fraction immediately adjacent to the origin (Cathode I) contained the greatest ACTH activity, but little of the intermedin activity.

Another experimental evaluation employed was the determination of the effect on adrenocorticotropic activity of conditions known to activate intermedin(10). In these experiments 3 mg of an ACTH preparation was dissolved in 10 ml of 0.1 N NaOH. The solution was placed in a boiling water bath for 5 minutes, and then immediately neutralized and cooled. A control solution was treated identically, except that the heat treatment was omitted. Both solutions were then diluted and simultaneously assayed for intermedin and adrenocorticotropic activities at the same dose levels. The results are shown in Table IV.

Potentiation of intermedin activity has apparently occurred. The control solution was negative at the 0.5 and 1.0 μg levels, whereas the boiled preparation gave a 2+ response at as low a level as 0.5 μg. Under these conditions, however, marked inactivation of the adrenocorticotrophic hormone took place, and only a small percentage of its original activity survived the alkali-heat treatment.

The last approach to the determination of ACTH and intermedin association involved assay for each of these activities in the separated anterior and intermediate lobes of the pituitary of the bullfrog, *Rana catesbiana*. With the frog under ether anesthesia, and dissection performed under the binocular microscope, a clear separation of these 2 lobes was attained. The lobes were frozen on dry ice immediately upon removal from the donor, then homogenized in 0.9% saline and assayed for the respective concentrations of the 2 activities.

Per unit of wet weight, the adrenal ascorbic acid depleting activity of the anterior lobes was at least twice that of the intermediate lobes, whereas the melanophore expanding activity of the intermediate lobe was approximately 60 times as great as that of the anterior lobe. Thus, the m.e.d. of the anterior lobes in this latter test was 700 μg, while the m.e.d. of the intermediate lobe was 12 μg. When considered in conjunction with the difference in adrenal ascorbic acid depleting activities, the relative difference between the activities in the 2 lobes is of the order of 100 times.

Discussion. The bulk of the data presented above is interpreted as a refutation of the claims of Johnsson and Högberg(1) and of Sulman(2,3) who purport to demonstrate the identity of ACTH and intermedin. Sufficient variation in intermedin activity has been found among a number of ACTH preparations, all of which were prepared from whole sheep pituitaries, to indicate that correlative studies are necessary. None of the aforementioned investigators has presented data demonstrating a correlation between the 2 activities.

The best demonstration of the non-identity of ACTH and intermedin would have to come from an actual chemical separation of the 2 hormones. Some success in this direction has been reported here with the use of a chromatographic procedure and filter paper electrophoresis. It is not here claimed that an ACTH preparation has been obtained in this laboratory which is free of melanophore expanding activity, but present evidence indicates that such a separation can be achieved. Raben, Rosenberg, and Astwood(11) have also recently reported an effective separation of the 2 activities.

Charcoal columns give a highly purified ACTH preparation, but the intermedin activity of such preparations is also greatly enhanced. As an isolated example, the activity of such preparations would lend support to the claims of a connection between the 2 activities.

It should be pointed out that the method of separation attempted by Johnsson and Högberg(1), *i.e.*, paper chromatography using phenol-water as a solvent, is recognized to be a system of limited usefulness for the purification of ACTH(12). Johnsson and Högberg (1) reported in addition that both hormones are inactivated by nitrite, formaldehyde, trypsin, and various tissue homogenates. These criteria cannot be considered sufficiently specific to characterize any polypeptide (see, for example, the effect of some of these reagents on posterior lobe principles(13)). On the other hand, potentiation of intermedin activity by heat treatment in an alkaline medium has effectively demonstrated the differing sensitivity of the 2 hormones to such a procedure, for little of the ACTH activity is retained after treatment.

An unequal distribution of the 2 hormones can also be demonstrated in the lobes of the pituitary gland by assaying the separated excised anterior and intermediate lobes of the frog. Determination of the concentration of both activities in each lobe has shown their non-identity.

It would appear that most of the presently available methods employed to purify ACTH also purify intermedin. Thus, the ACTH and pitressin preparations employed by Sulman possess more melanophore expansion activity than does his intermedin preparation(2). It may be noted that, in our experience, as little as 8 milliunits of pitressin (Parke-Davis) has given a positive intermedin response. Such preparations possess about 0.003 I.U. of ACTH activity per 10 I.U. of pitressin activity(14). Sulman(3) has found that a similar preparation of pitressin has an m.e.d. for intermedin activity of 500 milli-units. Furthemore, it can be speculated that most intermedin preparations must be heavily contaminated with ACTH, as evidenced by the ACTH-like clinical and physiological activities reported by Johnsson and Högberg(1), following the administration of such preparations to patients.

Summary. The validity of claims reporting an identity of ACTH and intermedin is questioned by experiments in which assays by the melanophore expanding test in the hypophy-

sectomized frog and the adrenal ascorbic acid depleting test in the hypophysectomized rat were employed. The results demonstrate: 1. Lack of correlation between the 2 activities in various ACTH preparations. 2. Partial separation of intermedin and ACTH activities using a discontinuous pH gradient on cellulose. 3. Differential migration of activities during zone electrophoresis on filter paper. 4. Potentiation of intermedin and inactivation of ACTH by alkali-heat treatment. 5. Differential concentration of ACTH and intermedin activities in the anterior and intermediate lobes of the frog pituitary.

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Effect of Various Allergens and Antibiotics on Experimental Poliomyelitis.* (19651)

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Last year we reported that the inoculation of pertussis vaccine, diphtheria toxoid or *Salmonella typhimurium* vaccine would significantly decrease the incubation period before onset of paralysis in mice infected with the Lansing strain of poliomyelitis virus(1). Since then we and others(2,3) have been able to confirm these results. Dean *et al.*(3) reported that mice injected in a foreleg with pertussis vaccine alone or combined with diphtheria-tetanus toxoids were more frequently paralyzed in the injected limb following infection with mouse encephalomyelitis virus than the unvaccinated controls. The present studies were carried out to determine the effect of various allergens and antibiotics on the incubation period of mice infected with the Lansing strain of poliomyelitis virus. The allergens tested included house dust, mixed tree pollen, mixed grass pollen, ragweed pollen, mixed mold spores, and horse serum. The antibiotics tested were dihydrostreptomycin, terramycin, and penicillin.

Materials and methods. Groups of Swiss mice (Plymouth strain) weighing 13 to 15 g were inoculated intracerebrally with approximately 10 LD_{50} units of the Lansing strain of poliomyelitis virus. The identity of the Lansing strain used in this laboratory was verified periodically by neutralization tests with hyperimmune Lansing antiserum. Control mice inoculated with nutrient broth were also included in every experiment. The various allergens tested were obtained through the courtesy of the Allergy Laboratory of Michael Reese Hospital and were used in the following concentrations: 1. House dust (Endo) 1:400, 2. Mixed tree pollen 1:500, 3. Mixed grass

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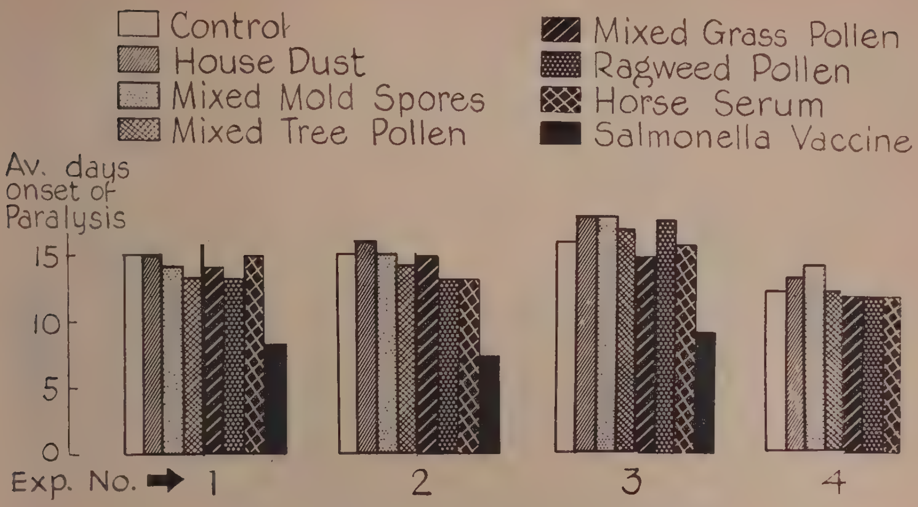

FIG. 1. Effect of various allergens on poliomyelitis.

pollen 1:500, 4. Ragwood pollen 1:33, 5. Mixed mold spores 1:200. The mixed mold spores contained species of the following genera: *Penicillium, Alternaria, Hormodendron, Aspergillus, Rhizopus, Mucor, Fusarium, Monilia,* and *Thoma*). The above dilutions of the various allergens studied represent the maximum concentrations that are usually employed for desensitization of patients. It was found that 0.1 cc of these dilutions inoculated intravenously were not toxic for the strain of mice used, although 10 times this concentration was often a lethal dose. The horse serum was used undiluted and was obtained from the Difco Laboratories. The *Salmonella typhimurium* vaccine was prepared as described previously(1). The antibiotics studied were dihydrostreptomycin, terramycin and penicillin. Streptomycin and terramycin were inoculated in 0.5 mg amounts dissolved in 0.1 cc of saline and given intravenously. This dosage was nontoxic and was approximately equivalent to the daily human dose on a weight basis. Thirty thousand units of procaine penicillin G in peanut oil was given intraperitoneally; in other experiments the same dosage of the crystalline form of penicillin G was given intravenously. The amount of penicillin given was approximately 1/10 the human daily dose.

The general plan followed in each experiment was as follows: The mice were divided into test and control groups. All animals were inoculated with approximately 10 LD_{50} units of the Lansing strain of the poliomyelitis virus. The same pool of Lansing virus was used in all experiments. As previously(1) on the first, third and fifth days after virus inoculation the various allergens and antibiotics in the above dosages were inoculated in 0.1 cc amounts intravenously or intraperitoneally. Control mice were inoculated with nutrient broth intravenously (0.1 cc) after the same intervals. Other control mice were simultaneously inoculated intravenously with 0.1 cc of *S. typhimurium* vaccine (approximately 100 million organisms) and showed little or no reaction.

Results. Allergens. Results obtained in a series of 4 experiments with over 300 mice are given in Fig. 1. The figure shows the average incubation period in days after virus inoculation for onset of paralysis in mice inoculated with the various allergens as well as control mice inoculated with nutrient broth or *Salmonella* vaccine. At least 20 mice were inoculated with each allergen, nutrient broth or *Salmonella* vaccine in every experiment. No mice were inoculated with *Salmonella* vaccine in Exp. 4, however, because of the difficulty in obtaining mice at the time that this experiment was carried out. The range of incu-

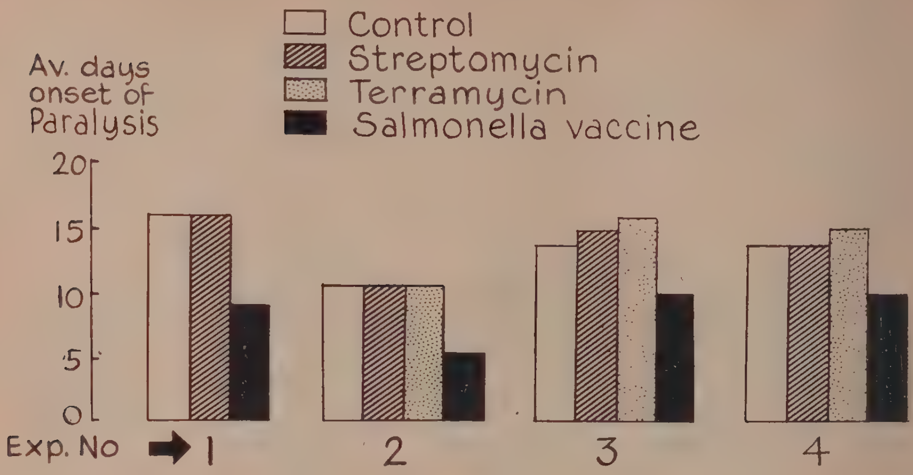

FIG. 2. Effect of various antibiotics on poliomyelitis.

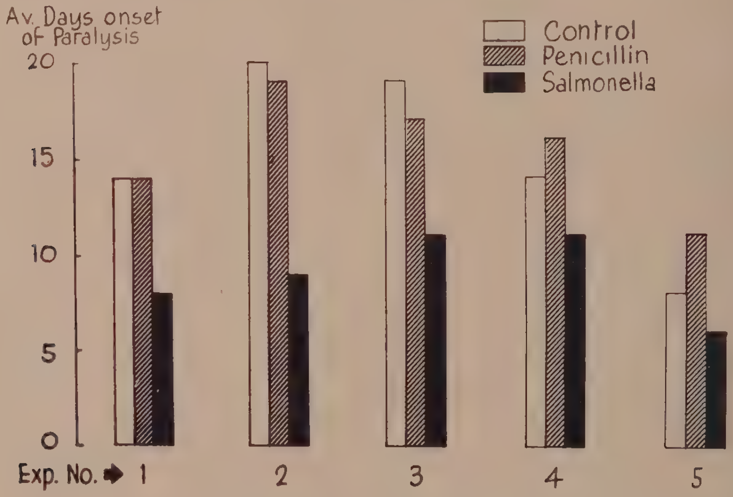

FIG. 3. Effect of penicillin and *Salmonella* vaccination on poliomyelitis.

bation period in both the test and control groups varied from 3 to 30 days. The graphs in Fig. 1 show that there was no significant reduction in the average incubation period before onset of paralysis in all experiments in which mice were inoculated with the various allergens or nutrient broth. In those experiments in which the *Salmonella* vaccine was included there was a consistent reduction in incubation period before onset of paralysis in this group. Statistical analysis of the data using the Student-Fisher t test as previously (1) indicated that there was no significant difference between the groups of mice receiving the various allergens and the controls inoculated with nutrient broth, while the reduction in incubation period following *Salmonella* vaccine inoculation was consistently significant.

Antibiotics. Figs. 2 and 3 summarize the results obtained in the average incubation period before onset of paralysis in infected mice inoculated with streptomycin, terramycin, and penicillin. All of the antibiotics were inoculated intravenously except procaine penicillin G which was given intraperitoneally. In other experiments the same dosage of crystalline penicillin G was given intravenously with similar results. Again at least 20 mice were inoculated with each antibiotic, nutrient broth or *Salmonella* vaccine. Approximately 500 mice were studied in these experiments. Statistical analysis by the t test showed that there was no significant difference between the groups of mice inoculated with the various antibiotics and controls inoculated with nutrient broth, while the *Salmonella* vaccine consistently produced a significant reduction in incubation period before onset of paralysis.

Discussion. These experiments indicate that inoculation of various allergens and antibiotics do not significantly decrease the incubation period before onset of paralysis in mice infected with the Lansing strain of poliomyelitis. These results are in contrast to those previously reported in which it was shown that inoculation of pertussis vaccine, pertussis-diphtheria toxoid or *Salmonella typhimurium* vaccine show a significantly decreased incubation period before onset of paralysis(1).

It is of interest that clinically there is a more suggestive relationship between inoculation of pertussis vaccine and/or diphtheria toxoid and paralysis in the inoculated limb than in the inoculation of various allergens and antibiotics during an epidemic of poliomyelitis(4-6). This relationship appears to be valid for the most part only if the inoculations are given 7 to 21 days prior to onset which corresponds to the usual incubation period of poliomyelitis. In a personal communication, Abramson(7) states that he conducted a survey of 800 physicians in the United States and collected data concerning 154,000 patients who received pollen injections before and during the hay fever season. Thirty cases of poliomyelitis occurred in the 154,000 patients receiving pollen therapy. "This incidence was considered about the normal rate for the country as a whole. Although only a small number of cases could be followed, there did not seem to be any correlation between injections of pollen and paralysis in the injected limb."

Summary. Mice infected intracerebrally with the Lansing strain of poliomyelitis virus show no significant decrease in incubation period before onset of paralysis following intravenous or intraperitoneal inoculation of certain allergens and antibiotics. The allergens studied include house dust, mixed mold spores, mixed tree pollen, mixed grass pollen, ragweed pollen, and horse serum. The antibiotics studied were dihydrostreptomycin, terramycin, and penicillin. It was found that *Salmonella typhimurium* vaccine significantly decreased the incubation period before onset of paralysis.

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Increased Excretion of Dehydroascorbic and Diketogulonic Acids by Rats in the Cold.* (19652)

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Recent work indicates that dehydroascorbic acid (DHA) and diketogulonic acid (DKA) are not major metabolic products of ascorbic acid (AsA) breakdown in the guinea pig(1). Normally only trace amounts of DHA and DKA occur in animal tissues. When large doses of AsA are given, the increase in these oxidation products in the animal body is only a fraction of the amount which would be expected if most of the AsA were metabolized by conversion to DHA(1). The concept that AsA is utilized in the animal body as a component of some oxidation-reduction system has been frequently suggested, despite the lack of experimental evidence for employment of such a system. Numerous investigators have confirmed the fall in adrenal AsA after exposure of the intact animal to stress, following the initial reports of Sayers *et al.* on this phenomenon(2,3). Other observations have indicated AsA supplementation increases the ability of the animal to withstand stress(4), and Therien and Dugal have reported increased excretion of AsA in rats exposed to the stress of a cold environment(5).

Nearly all of the reported changes in AsA tissue levels or urinary excretion have been based on methods of analysis which do not differentiate AsA from DHA and DKA. Methods depending upon the reducing power of AsA, such as the Tillmans method and adaptations made from it(6) measure only AsA. Methods which depend upon the coupling of oxidized AsA with dinitrophenylhydrazine, such as the Roe-Kuether method(7), measure AsA, DHA, and DKA without differentiation. This report shows there is a marked change in urinary excretion of DHA and DKA, as well as of AsA, when the 3 components are determined by a method which allows analysis of each(8).

Experimental. Young albino rats, equally divided as to sex, with starting weights of about 200 g, were housed in individual metabolism cages, and fed Purina chow *ad lib.* Drinking water containing 5% ethanol as an antifreeze was provided to both control and experimental animals. The floors and funnel portions of the cages were coated with a silicone product to prevent the voided urine from coming in contact with the metal. The urine was collected into beakers containing 0.5 g $SnCl_2$ in 10% HPO_3. The cages were washed down each day and the urine and washings diluted to 200 ml with 10% HPO_3 to make a final concentration of 5% HPO_3, and analyzed for AsA, DHA, and DKA(8).

The experiments were divided into 2 series; in series I the control values were determined before the period of exposure to cold environment; in series II the controls were obtained after the experimental period. The control values were obtained while housing the animals in a room with the temperature controlled at 21°C ± 1°C, and the experimental values after placing the animals into a deep freeze box at 0°C ± 0.5°C. This box was outfitted with an intake air hose, and an outlet hose attached to negative pressure, assuring a constant flow of fresh air through the box. The excretion values for each rat were followed for about 7 days at 21°C, and 10 days at 0°C.

The animals showed great variation in daily excretion of AsA and the 2 oxidation products, both at normal and cold environmental temperatures. Table I shows the results of Series I. The excretion of AsA in the cold is 66% greater than the control excretion; that of DHA is 300% greater, and DKA is 500% increased. Table II shows the results from Series II. The values for DHA excretion did not return to control levels immediately, and the mean change in this fraction is less than in

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* Supported by a grant from the USAF School of Aviation Medicine, Randolph Field, Texas.

TABLE I. Urinary Excretion of Ascorbic, Dehydroascorbic and Diketogulonic Acids by Rats at Normal and Cold Environmental Temperatures.

Fraction determined	No. of determinations 21°C	0°C	Avg 24-hr excretion in µg with S.D. 21°C	0°C	F
AsA	61	80	574 ± 661	955 ± 1235	4.79*
DHA	61	80	65 ± 66	271 ± 198	61.9 †
DKA	61	80	38 ± 62	243 ± 187	66.2 †

* Probability of variance ratio "F" less than .05, greater than .01.
† " " " " " " " .01.

TABLE II. Urinary Excretion of Ascorbic, Dehydroascorbic and Diketogulonic Acids by Rats at Cold and Normal Environmental Temperatures.

Fraction determined	No. of determinations 0°C	21°C	Avg 24-hr excretion in µg with S.D. 0°C	21°C	F
AsA	91	66	704 ± 725	500 ± 706	3.14
DHA	91	66	157 ± 170	82 ± 79	11.3 †
DKA	91	66	227 ± 181	39 ± 66	66.4 †

† Probability of variance ratio "F" less than .01.

TABLE III. Urinary Excretion of Ascorbic, Dehydroascorbic and Diketogulonic Acids by All Rats at Normal and Cold Environmental Temperatures.

Fraction determined	No. of determinations 21°C	0°C	Avg 24-hr excretion in µg with S.D. 21°C	0°C	F
AsA	127	171	536 ± 677	821 ± 995	7.75†
DHA	127	171	74 ± 72	211 ± 190	59.1 †
DKA	127	171	39 ± 63	234 ± 182	133 †

† Probability of variance ratio "F" less than .01.

Series I. In this series the AsA difference does not reach the 5% level of significance, but when both series are considered together, the greater number of degrees of freedom increases the "F" value for all fractions beyond the level of high significance, as shown in Table III. The increases for the combined values of AsA excretion in the cold average 53% more than the control excretion; DHA is 186% greater; DKA, 500% greater.

Discussion. It is interesting to speculate whether this observed increase in the excretion of oxidized AsA by the rat during exposure to low environmental temperature is the result of some mechanism in the animal body which utilizes the reducing capacity of AsA as a means of adjustment to cold stress. This is the first direct evidence that the oxidized forms of AsA may be normal metabolic products in the rat. A great many of the reported changes in AsA in the animal body might profitably be reinvestigated to ascertain whether there is a concomitant change in DHA and DKA as well.

Summary. 1. Adult albino rats were exposed to environmental temperatures of 21°C for control, and 0°C for experimental periods. 2. Urinary excretion of ascorbic acid at 0°C was increased 53%, dehydroascorbic acid increased 186%, and diketogulonic acid 500% above the values obtained on the same rats at 21°C. 3. All of these increases were found to be highly significant.

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Persistence of C^{14}-Anthranil-azo-ovalbumin in Injected Rabbits.[*] (19653)

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The organ deposition and intracellular distribution of heavily radio-iodinated antigens following their injection into rabbits has already been reported(1-4). Iodine 131 suffers from certain disadvantages as an antigen label in *in vivo* experiments. Iodoproteins, due to their similarity to the thyroid hormone, could conceivably be metabolized in a manner not representative of antigens in general. Moreover, the short half-life of I^{131} renders experiments of very long duration impossible. Both of these difficulties were avoided in the present investigation by coupling the protein antigen with diazotized 7-C^{14}-anthranilic acid.

*Experimental. Preparation of 7-C^{14}-anthranil-azo-ovalbumin (A*AO).* Crystalline ovalbumin was prepared from hen's eggs by the method of Kekwick and Cannan(5). All operations for the preparation of A*AO were carried out in the cold, using chilled solutions. In a typical preparation, 33 mg 7-C^{14}-anthranilic acid,[§] containing 27 μc per mg, was dissolved in 2.7 ml of 0.2 *N* HCl, and diazotized by adding 0.05 *N* sodium nitrite (4.5 ml) until there developed a positive test with starch and iodide. The solution was poured into 7.5 ml of a solution containing 3.3% sodium carbonate and 3.9% ovalbumin. Coupling was accelerated by the subsequent addition of 0.95 ml *N* NaOH. After standing over night, the solution was dialyzed against distilled water, precipitated by dilute hydrochloric acid, centrifuged, and the precipitated azoprotein suspended in water and dissolved by adding sodium carbonate to pH 7. A large amount of a non-radioactive preparation was prepared parallel with the radioactive one. The 2 preparations were mixed in various ratios to afford solutions for injection of the desired specific activity. Spectrophotometric comparison of our anthranil-azo-ovalbumin with anthranil-azo-tyrosine at a wave length of 460 mμ indicated 11.8 azo groups per protein molecule, assuming a molecular weight of

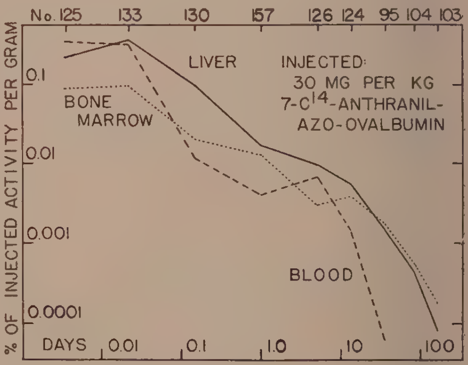

FIG. 1. Antigen content of blood, liver, and bone marrow after the injection of C^{14}-anthranil-azo-ovalbumin to rabbits. The number of each rabbit is recorded at the top of the figure; the vertical lines under the numbers indicate the time of killing; the time scale, at the bottom of the figure, is logarithmic. Analyses of other organs and of subcellular fractions of the same rabbits are recorded in Table I.

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[§] Supplied by the Oak Ridge National Laboratories.

TABLE I. Organ Levels and Intracellular Distribution in Tissue Proteins Following a Single Intravenous Injection of 30 mg/kg 7-C^{14}-anthranil-azo-ovalbumin (A*-Ov).

Rabbit No.	125	133	130	157	126	124	95	104	103
Time killed	5 min	30 min	3.5 hr	23.7 hr	4.7 d	11.8 d	33 d	79 d	148 d
Wt (kg)	1.6	2.2	2.2	1.5	1.9	1.8	2.9	2.9	2
Liver wt (g)	89	83	110	82	75	84	109	77	99
A*-Ov (%)†	18.2	28	10.7	1.35	.83	.50	.185	.040	.0073
Organ:	% of inj. activity/g whole tissue × 1000:								
Spleen	233	300	238	24.8	27.4	15.2	2.95	.90	.39
Lung	1780	139	26.5	3.06	8.41	1.64	.18	.096	—
Kidney	82	91	20.6	12.5	4.3	4.5	2	.6	.2
Heart	48	55	11	1.6	(10?)	1.2	.05	—	—
Liver fraction:	Wt of TCA-precipitates derived from a g of whole liver (mg):								
Nuclear	23.7	42.5	21.1	30.1	32.1	53.5	27.4	44.6	28.2
Mitochondrial	27.1	25.5	22.2	24.5	27	29.9	23.7	25.1	24.6
Microsomal	20.3	18.5	20.3	22.2	22.6	30.6	29.1	27.4	21.1
Supernatant	44.8	65.2	39.2	41.5	47.3	71	63.8	67.8	—
Liver fraction:	Relative activities:*								
Nuclear	1.49	.80	.87	1.60	1.07	.91	1.43	1.12	.82
Mitochondrial	1.45	3.05	2.57	1.77	1.84	2.18	3.65	3.40	4.1
Microsomal	1.46	1.31	.72	.38	.45	.67	.17	.71	2.3
Supernatant	.22	.22	.29	.51	.73	.61	.16	.15	.0
Spleen fraction:	Relative activities:*								
Nuclear	.54	.59	.66	.78	.58	.89	1.19	1.30	1.71
Mitochondrial	2.94	3.72	4.23	2.38	2.21	2.80	2.69	2.64	1.18
Microsomal	3.68	1.38	.89	1.53	3.45	1.12	1.12	.29	.0
Supernatant	.45	.44	.18	.72	.76	.42	.42	.12	.0

* Activity of total homogenate ≡ 1.

† In % of injected radioactivity per whole liver.

45000 for ovalbumin. Recovery of anthranilic acid radioactivity in the azoprotein indicated 11.9 residues per protein molecule.

Preparation of samples and counting of radioactivity. The experimental procedures with regard to sacrifice of the rabbits and the preparation and centrifugal fractionation of the organ homogenates were essentially those already described in detail(4). All injections were made intravenously into the marginal ear vein and consisted of 30 mg A*AO per kg of body weight. The livers of the rabbits were perfused with about 500 ml of chilled isotonic sucrose (0.25 *M*) via the portal vein. Homogenates were prepared using 0.88 *M* sucrose solution. The bone marrow was taken from the femur. Organ homogenates, fraction suspensions, and standard preparations were precipitated with trichloroacetic acid, and the precipitates washed as described previously (4). Radioactivity was measured in a Q-gas flow counter, or with a thin mica window Geiger tube. To correct for self-absorption, the counting rate of each sample was recalculated for a sample thickness of 8.1 mg/cm^2, using a calibration curve in which the activity of a C^{14}-containing protein precipitate was plotted against the sample thickness.

Results. Persistence of C^{14}-anthranil-azo-ovalbumin in organs. Fig. 1 and Table I give the data for organ deposition of trichloroacetic acid precipitable radioactivity following the injection of A*AO. In Fig. 1, the percentage of injected radioactivity per g of whole liver and bone marrow, and per ml of blood is plotted on a logarithmic scale against the time elapsing between injection and sacrifice, also plotted on a logarithmic scale. Similarly, tabulated data for lung, kidney, and spleen are given in Table I.

Intracellular distribution of deposited anthranil-azo-ovalbumin in liver and spleen. Table I also describes the distribution of radioactivity among the trichloroacetic acid precipitates of the subcellular fractions of liver and spleen, isolated from homogenates of these organs by differential centrifugation. The relative activities for liver were obtained by dividing the counts per minute per mg protein of a fraction, by the counts per minute per mg of the whole homogenate protein. The relative activity of the whole homogenate is therefore 1.0. and the value for the several fractions tell how many times more concentrated the radioactivity is in the proteins of a particular fraction than in the proteins of the whole homogenate. The values for spleen were obtained by dividing the percent of the radioactivity residing in each spleen fraction, by the percent of the total spleen nitrogen it accounts for. The average spleen nitrogen distribution figures previously used(4) were employed.

Discussion. The results obtained here with C^{14}-anthranil-azo-ovalbumin in general parallel those obtained with I^{131}-iodo-ovalbumin(4) and arsanil-azo-proteins(6). From Fig. 1 and Table I it is apparent that the antigen is cleared rapidly from the blood, and that it is detectable in spleen, bone marrow, liver and kidney for several months. After long periods of time it is most concentrated in spleen and bone marrow. The persistence of anthranil-azo-ovalbumin in rabbit organs is in agreement with the long-term storage of azoproteins in the tissues of mice reported by McMaster and Kruse(7).

When injected *in vivo,* the antigen undergoes a brief initial concentration in the microsomal fraction of liver and spleen (Table I), and possibly of other organs as well. After a short time, however, the antigen is found chiefly in the mitochondrial fraction, where it appears to remain concentrated over long periods of time (Table I). The accumulation of the antigen in the cytoplasmic granules is not simply a non-specific adsorption effect; when the chilled liver or spleen homogenates from normal rabbits are mixed *in vitro* with the antigen, and 30 minutes later fractionated, or when the mixture of antigen and homogenate is incubated at 37°C for 15 minutes, the bulk of the radioactivity is recovered in the final supernatant (Table II, Nos. 140 and 151). The same result was obtained when homogenate No. 151 was incubated with the added antigen at 37°C for 30 or 45 minutes before beginning the fractionation.

In previous experiments with arsanil-azo-protein there remained the possibility that a portion of the arsenic found in the tissues was metabolized inorganic arsenic(6). The present experiments are free from this objection since we measured only trichloroacetic acid precipitable radioactive carbon.

TABLE II. Relative Activities of the Proteins of Subcellular Fractions after Addition of 7-C^{14}-anthranil-azo-ovalbumin *In vitro* to Homogenates of Normal Rabbit Organs.

Rabbit No.	140	140	151
Organ	Liver	Spleen	Liver
Antigen added (μg/g tissue)	95	119	95
Incubation: time (min)	30	30	15
temp. (°C)	<5	<5	37
Relative activity:			
Nuclei	.47	.31	.61
Mitochondria	.19	.32	.28
Microsomes	.28	.45	.32
Supernatant	1.86	2.75	1.68

The question arises as to whether all of the protein-bound C^{14} is present as unchanged antigen, or whether some of the radioactive anthranilic acid or anthranil-azo-tyrosine was split off and was recombined with insoluble tissue proteins. Although the formation of free anthranilic acid from anthranil-azo-ovalbumin and its incorporation into other proteins is highly improbable, we investigated this possibility by injecting into a rabbit (No. 156) C^{14}-anthranilic acid in an amount equivalent to 30 mg anthranil-azo-ovalbumin per kg body weight. The animal was killed after 24.5 hours. The radioactivity of the liver and blood proteins was 0.00016 and 0.00023% of the injected activity per g liver or ml blood, respectively; evidently, injected anthranilic acid was not deposited in the liver. Analysis of the urine of this animal showed almost quantitative excretion of the injected radioactivity through the kidney. When an equivalent amount of C^{14}-anthranil-azo-ovalbumin was injected into rabbit No. 157, 0.0165% of the injected radioactivity was found in one g liver, *i.e.,* 100 times more than after the injection of anthranilic acid. These results render unlikely the metabolic incorporation of C^{14}-anthranilic acid, split off from the injected azoprotein, as an explanation for the lengthy persistence of C^{14} in the tissue proteins. This is in accordance with observations of Kruse and McMaster(8) who have shown that free azo dye, in contrast to azo-protein, is not deposited in the organs, but is rapidly excreted.

Since the radioactivity of our antigen molecules is localized in the determinant *o*-azobenzoic acid groups which are responsible for the serological specificity, our analyses reveal only the fate of these groups, not that of the ovalbumin moiety which, in our experiments, acts only as non-specific carrier of the determinant groups. Although the transfer of radioactive anthranil-azo-peptides from deposited antigen molecules to the proteins of the body cells is rendered unlikely by the experiments mentioned in the preceding paragraph, we cannot exclude this possibility. However, it is well known that low-molecular "proantigens" such as acyl chlorides(9) or oxazolones(10) are converted into true antigens by combination with proteins of the injected animal. The formation of anthranil-azo-tissue proteins would not affect, therefore, our view that the antigen, or, more precisely, its serologically active specific group, is stored in the body cells over long periods of time.

The question may be raised, whether the radioactivity of the tissue proteins is not due to the metabolic incorporation of radioactive carbon dioxide derived from the breakdown of anthranilic acid residues of the antigen. This is unlikely for the following reasons: I) After 24 hours, less than 10% of the injected radioactivity remains in the rabbit; the small residual amount of antigen is slowly degraded, and any carbon dioxide derived from it is diluted with a large excess of non-radioactive carbonic acid from the bicarbonate pool of the body; since most of the carbonic acid is excreted through the lung and the urine, only traces would be available for incorporation into body constituents. II) The ratio (C^{14} in heart muscle protein)/(C^{14} in liver protein) decreases in our experiments from a value of 0.2 on the 12th day to 0.03 on the 33rd day after injection. If $C^{14}O_2$ were incorporated into the tissue proteins, one would expect the opposite phenomenon because the turnover of muscle protein is slower than that of liver protein. This was first demonstrated by Shemin and Rittenberg(11) who observed an increase in the ratio (N^{15} in muscle protein)/(N^{15} in liver protein) during the incorporation of N^{15}-glycine.

We conclude from all these considerations that formation of $C^{14}O_2$ and its incorporation into tissue proteins play no significant role in our experiments and that most of the radioactivity found in the tissue proteins after long periods of time, is present as 7-C^{14}-anthranil-azo-protein. Although the persistence of the antigen in the tissue does not constitute definitive proof that the presence of the antigen is necessary for antibody formation, our results support this view. They are difficult to reconcile with theories based on the assumption that antigen disappears rapidly from the organs and that antibody formation continues after elimination of all of the antigen(12).

Summary. At various intervals of time following a single intravenous injection of 7-C^{14}-anthranil-azo-ovalbumin to rabbits, the organ deposition and intracellular distribution in liver and spleen of trichloroacetic acid precipitable radioactivity was determined. The antigen was cleared rapidly from the blood and deposited in various organs, where significant amounts were retained for many months; in the initial moments of deposition, the antigen was concentrated in the microsomal proteins of liver and spleen; subsequently it was most concentrated in the mitochondrial proteins, where it remained stored indefinitely.

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II. Treatment of Flexner-Jobling Carcinoma in Sprague-Dawley Rats with 2,4,6-Triethylenimino-s-Triazine (TEM)*† (19654)

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We have previously reported(1) that the treatment of the King A rat bearing transplanted sarcoma 231 with suitable doses of 2,4,6-triethylenimino-s-triazine and related products resulted in complete regression of the tumor in the majority of the animals. These rats were then resistant to further transplants of the tumor.

It seemed important to know how different types of rat tumor would respond to treatment with the ethylenimines. From a preliminary investigation of tumor growth in mice it appeared that the compounds might have a greater retarding action on the growth of carcinomas than sarcomas(2). To see if this might be true of rat tumor, we have chosen to investigate first the response of the well known Flexner-Jobling carcinoma to therapy with 2,4,6-triethylenimino-s-triazine.

Materials and methods. Young Sprague-Dawley rats were used as carriers of the Flexner-Jobling carcinoma. The animals were selected for the particular experiment so as to secure maximum uniformity. The methods used were essentially the same as previously described(1). An isotonic saline solution containing the required dose of the drug in 0.1 ml was prepared each day and this dose injected intraperitoneally either twice or 4 times daily during the course of therapy. In all cases the treated rats were pair-fed with the controls. The rats were treated only after the carcinoma had established itself as a growing neoplasm. Treatment was started from 8 to 14 days after the implantation and the tumors averaged from 180 to 1000 mm^2.

Results. Eight young male rats, averaging 140 g, were implanted with the carcinoma. Four of the group served as controls and the other 4 were treated, beginning on the 8th day after the implantation, with 0.057 mg/kg of TEM 4 times daily. This treatment was continued for 4 days. Reversal of tumor growth took place in all 4 rats on the third day after treatment began and the regression continued to completion without further medication. The only toxic symptom observed was loss of appetite during the therapy. A few days after the drug was stopped the rats regained their normal appetite and went on to gain weight and develop normally. The tumors in the controls grew rapidly and ultimately resulted in the death of the animals. The tumors ulcerated in the untreated animals, as is usual for this tumor, but did not in the treated rats. The cured rats proved to be resistant to further transplants of the carcinoma.

Four young male rats of about 85 g gross weight, bearing 9-day tumors, were treated with 0.053 mg/kg of TEM twice daily, and this therapy continued for 18 days. The tumors regressed completely in 3 and partially in one. In this latter case, regression of the tumor proceeded for 7 days and then stopped; growth began again and continued at an accelerated rate in spite of the treatment.

Twelve young rats, averaging 170 g, were selected 11 days after implantation when the tumor size was about 700 mm^2; 8 were treated 4 times daily with 0.053 mg/kg of TEM and 4 maintained as untreated controls. This treatment continued for 4 days, during which time the rats received about the same amount of drug as had caused complete regression of the tumors in all the animals in the group bearing the 8-day tumor. From the previous results, no further treatment with the drug should have been necessary. However, after the period of therapy the tumor in but one animal showed a reversal of growth. Two days after the animal had been off the drug, regression of the tumor started in another rat of the group. The rate of growth of the tu-

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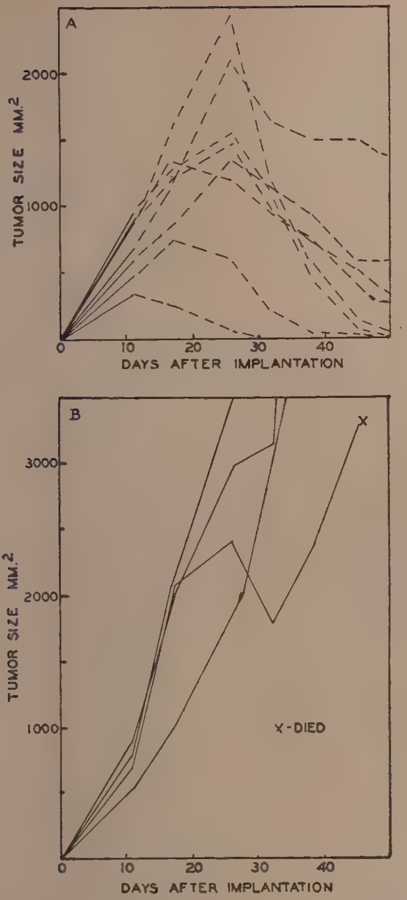

FIG. 1 A. Growth curves of the carcinoma in young rats treated 4 days with .053 mg/kg of TEM 4 times daily; 3 days no treatment and 34 days treatment with .053 mg/kg of the drug twice daily. The solid portions of the curves indicate the periods of no treatment.

B. Growth curves of the carcinoma in the untreated controls, pair-fed with 1 A.

mors in the other 6 rats indicated little or no retardation by the treatment. One day later the therapy was resumed, but with only 2 daily injections. This was continued for 34 days, during which time 4 tumors regressed completely; one having reached a size of 2400 mm^2 before growth stopped and regression began. Regression of the tumor in a fifth animal in the group was continuing when the experiment was terminated; 52 days after implantation, while in the other 3 animals the tumor size appeared to be contained at the lowest level reached. All of the animals were in good condition. Those with tumors were sacrificed for histological studies. The animals in which the tumors regressed were later tested and found to be resistant to further transplants of the tumors. They were cured. All of the untreated controls died of the carcinoma between 34 and 49 days after the implantation. The comparative growth of the tumors in the treated and untreated rats is illustrated in Fig. 1A and Fig. 1B.

Fourteen male rats averaging about 270 g and bearing 14-day tumors of 650 mm^2 average size were selected for a comparative study with the doses of TEM which we used most effectively in the treatment of sarcoma 231. Four animals received 0.016 mg/kg; 4, 0.023 mg/kg; and 2 animals, 0.046 mg/kg twice daily, during the entire course of treatment, lasting 29 days. The remaining 4 animals were untreated and served as controls. There was also a group of 4 normal controls pair-fed with the animals bearing the tumor in order to check on the adequacy of the food intake. The treated animals in which the tumor regressed completely and the normal controls gained weight. There were 2 complete regressions in the first group, 3 in the second, and 2 in the third. In the group receiving the smallest amount of drug, the tumors in 2 of the animals continued to grow at about the same rate as before treatment until 15 days of therapy, when they stopped growing and regressed for the next 5 days; then, growth was continued for the remaining 9 days of treatment. The therapy was discontinued and soon thereafter the tumors began to grow again and the growth continued at an accelerated rate until the carcinoma killed the animals. However, these animals lived 64 days while all untreated controls were dead by the 28th day. In a group of 4 animals treated with 0.023 mg/kg twice daily, the tumor had regressed completely in 2, almost to completion in one, and only slightly in the other when the therapy was discontinued. Both of the existing tumors continued to regress without further therapy; the small one to completion and the larger one to about half its size. At this stage the growth of the

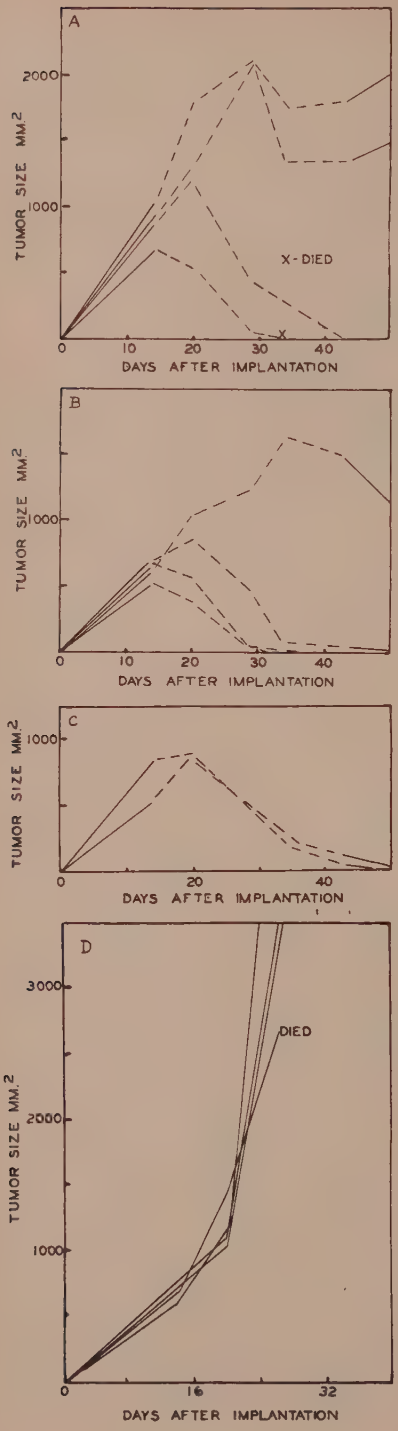

FIG. 2 A. Growth curves of the carcinoma in rats treated with .016 mg/kg TEM, twice daily. The solid portions of the curves denote the periods of no treatment.

B. Growth curves of the carcinoma in rats treated with .023 mg/kg of TEM, twice daily. The solid portions of the curves indicate the periods without treatment.

C. Growth curves of the carcinoma in rats treated with .046 mg/kg of TEM, twice daily. The solid portions of the curves represent the periods of no treatment.

D. Growth curves of the carcinoma in rats untreated and serving as controls for 2 A-2 C.

latter began again and continued at a moderate rate. The animal lived more than 64 days, showing that treatment had helped to hold in check the killing power of the tumor. The tumor in both of the animals treated with 0.046 mg/kg twice daily regressed to small size during the period of treatment and continued to complete regression after the therapy was discontinued. The comparative effects of treatment with different doses are shown in Fig. 2A-2D.

There were 26 rats treated in the several groups at the different dose levels and complete regression of the tumor occurred in 18. The tumor did not regress in any of the 13 control animals.

The 18 rats in which the tumor was completely cured were all resistant to the growth of further transplants of the tumor and in no case did the tumor which had regressed reappear.

Several of these rats were sacrificed soon after the third challenge for immunity and their organs and cavities examined. No tumors or lesions were found. About 6 months later another group was sacrificed and examined with similar results. The remaining animals were alive one year after being cured of the tumor. They were normal in weight and well-being. They were sacrificed along with the controls that had survived and a comparative examination made. No tumors or lesions were found in either group.

Discussion. The Flexner-Jobling carcinoma in the Sprague-Dawley rat responded to treatment with triethylenimino-s-triazine over a wider range of doses than sarcoma 231 in the King A rat. In the latter no regressions were obtained with 0.2 mg/kg/D either in a short or long course of treatment and the best results were obtained with about 0.04 mg/kg/D. Regressions of the carcinoma have been obtained with 0.032 to 0.230 mg/kg/D and without toxic manifestations by the hosts.

In general this carcinoma ulcerates in its later stages of growth. However, in the treated animals this occurred less often. In most of the cases where regression started early in the treatment it proceeded to completion without ulceration of the tumor.

It would appear that the Sprague-Dawley rat is less sensitive than the King A rat to TEM. The animals gained weight under treatment.

Summary. 1. The treatment of Sprague-Dawley rats bearing Flexner-Jobling carcinoma with 2,4,6-triethylenimino-s-triazine resulted in complete regression and cures of the tumor in about 70% of the animals treated. There were no spontaneous regressions among the controls. 2. This carcinoma responded to treatment with doses ranging from 0.016 to 0.046 mg/kg, injected twice daily for the entire course of treatment and the rats showed no marked symptoms of toxicity. 3. A short course of treatment with 0.230 mg/kg/D divided in 4 equal doses and given but 4 days, resulted in complete regressions of the tumors in all of the animals treated when the therapy was begun 8 days after the implantation but this method did not give equally good results in another group treated 11 days after implantation. In this case it was necessary to institute a second course of treatment with 0.052 mg/kg, twice daily for 34 days to obtain complete regression of the tumors in 50% of the animals. 4. All of the rats in which the tumor regressed completely were then resistant to further implants of the tumor. 5. Rats that were cured lived for more than a year without the tumor reappearing. In comparison with the controls they were normal. 6. No gross manifestations of toxicity were seen with the therapeutic doses of TEM used. 7. There was no evidence of carcinogenicity of TEM in the therapeutic doses used in the Sprague-Dawley rats up to one year after the end of the period of therapy.

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A Critical Examination of the Histochemical Staining Technic for Ascorbic Acid. (19655)

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The acid silver nitrate method for the identification and localization of ascorbic acid in tissues has been used extensively since its introduction by Gough and Zilva(1). Some workers(2-5) claim good specificity and precise localization, although they admit that a negative reaction does not exclude the presence of the vitamin. Others(6-8) do not consider precise localization to be possible by this technic, but offer no or inconclusive evidence for their contention.

In the study to be presented, the sensitivity, specificity, and localizability of the histochemical method for ascorbic acid have been investigated by test tube and model experiments and by the use of animal tissues.

Experimental. 1. A solution of acetic acid and silver nitrate was added to the test substances in centrifuge tubes. The precipitate was washed and the unreduced silver removed by thiosulfate or ammoniacal thiosulfate solution. A large number of substances was tested, and positive reactions (a precipitate of metallic silver) were obtained with ascorbic acid, 3,4-dihydroxyphenylalanine, hydroquinone, and a mixture of calcium and magnesium

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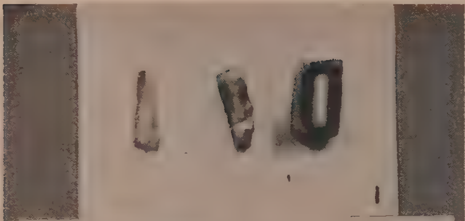

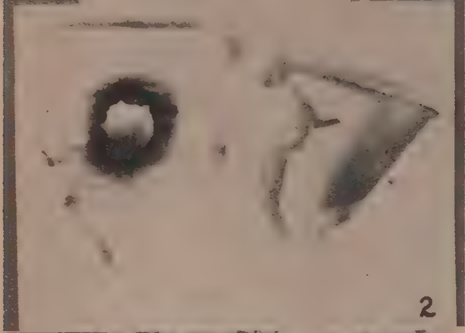

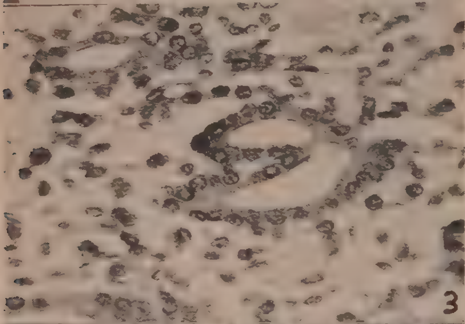

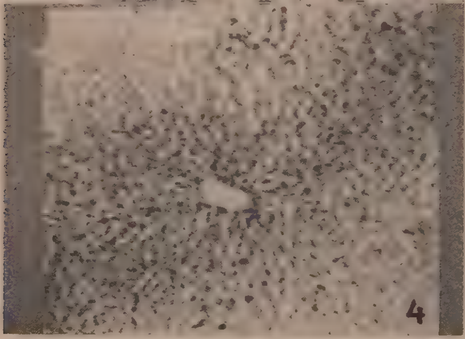

FIG. 1. Ascorbic acid-free gelatin blocks on which the acid silver nitrate histochemical reaction has been performed. Thickness of the blocks and duration of the pre-thiosulfate wash, from left to right, are: 1.5 mm and 30 min, 1.5 mm and 10 min, and 2.5 mm and 10 min. The thiosulfate artifact is demonstrated.

FIG. 2. Gelatin model with ascorbic acid present in the central core (removed) and absent from remainder at start of experiment, demonstrating diffusion artifact (ascorbic acid-free control on right).

FIG. 3. Normal guinea pig kidney, high power (45× oil immersion objective), paraffin section, demonstrating silver impregnation without visible granules.

FIG. 4. Supersaturated guinea pig liver, low power (10× dry objective), paraffin section, showing band of more intense silver deposition (Liesegang ring).

phosphates and carbonates, using neutral thiosulfate to remove unreduced silver. In addition, acid thiosulfate itself, when used as the test substance, was observed to precipitate metallic silver. Using ammoniacal thiosulfate to remove the excess silver nitrate, ascorbic acid, 3,4-dihydroxyphenylalanine, hydroquinone, glucose-6-phosphate, alpha-naphthol, and ribose produced a positive reaction.

2. The addition of gelatin to ascorbic acid solutions had 2 effects: a) the development of color due to reduced silver was greatly delayed; and b) the reduced silver tended to remain in a fine colloidal suspension instead of forming a granular precipitate.

3. The effect of glutathione has again been confirmed by means of spot plate reactions using various mixtures of ascorbic acid, gelatin, and glutathione. In addition to decreasing the sensitivity of the reaction, however, glutathione was observed to delay the appearance of a visible precipitate.

4. Formalin fixed, thoroughly washed gelatin blocks were placed in the acid silver nitrate reagent, washed, and "fixed" in thiosulfate. Unless washings were thorough, acid thiosulfate reduced the silver during the succeeding step (Fig. 1). The addition of ascorbic acid-free serum to the gelatin greatly increased the tendency to diffuse silver impregnation.

5. Holes bored in gelatin blocks were filled with ascorbic acid-carmine red-gelatin solution and the histochemical reaction applied. Diffusion was indicated by the precipitation of silver far beyond the limit of the ascorbic acid-containing central core (Fig. 2).

6. The acid silver nitrate reaction was applied to tissues from normal, scorbutic, and "supersaturated" guinea pigs, along with correlative biochemical studies(9). Fresh frozen, direct mounted sections(10) and tissue blocks

TABLE I. Comparison of Tissue Level of Ascorbic Acid with the Histochemical Reaction.

Organ	Concentration (mg %) Fresh frozen	Paraffin
Histochemically negative		
Adrenal	19	12.5, 9
Testis	16, 29	18
Kidney	15, 34	15, 26, 34
Liver	71, 36, 37	71, 33, 28
Histochemically positive		
Adrenal	127, 61	80, 61
Testis	45	45, 29
Kidney	214	217
Liver	105	109, 37, 33
Bone		2.6, 3, 31, 2.3

were used in the histochemical reaction. The data are listed in Table I. As shown in the table, bone is always silver impregnated when neutral thiosulfate is used following the reaction. Other silver artifacts are seen, including brown to black uniform impregnation of connective tissue of some kidney and testis blocks, the internal elastic lamina of the arteries of one animal, and basement membranes, cell walls, nuclear membranes, and even chromatin of liver, kidney, and testis of some animals (Fig. 3).

Many blocks of tissue showed waves of positive reaction suggesting the Liesegang ring phenomenon (Fig. 4). With fresh frozen sections, the reaction was weaker and more diffuse than with tissue blocks, and was often negative in thinner and positive in thicker areas.

Discussion. The test tube and model experiments and animal observations have been observed to essentially corroborate each other as enumerated below. 1. All bones, regardless of ascorbic acid content, retain silver ions tenaciously for gradual spontaneous and/or photoreduction. The mixture of calcium and magnesium phosphates and carbonates acted similarly in the test tube. 2. High concentrations of ascorbic acid are needed for the formation of a granular precipitate in the presence of tissue proteins as well as in the presence of gelatin. 3. The reduction of silver ions to metallic silver by acid thiosulfate is observed in both tissue and gelatin blocks when inadequately washed following the reaction. 4. Diffusion is prominent as demonstrated by the results with fresh frozen sections, by the waves of positive reaction (Liesegang rings) in tissue blocks, and by the models of Exp. 5. 5. Silver impregnation artifacts occur despite meticulous care in technic. 6. Although the use of ammonium hydroxide (11) to remove the excess silver salts following the reaction minimizes the bone artifacts, it greatly decreases the specificity of the reaction by converting to the more easily reducible alkaline silver nitrate.

Summary. The specificity, interfering artifacts, sensitivity, and localizability of the acid silver nitrate reaction for ascorbic acid were studied by means of test tube and slide reactions, model experiments, and histological methods. Whereas the specificity of the reduction reaction is reasonably good, interfering artifacts are numerous and are difficult to avoid. Protein and glutathione greatly reduce the sensitivity and velocity of the histochemical reaction. Gross diffusion artifacts render the test useless for localization on a cytological level.

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Antihistaminic Action of 'Pyronil'.* (19656)

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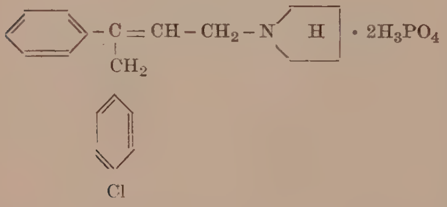
Continuous research for synthetic substances capable of inhibiting the physiological action of histamine has led to the preparation and study of numerous compounds possessing this property. The present goal in antihistaminic investigation appears to be the synthesis of a drug possessing no unpleasant clinical side reactions common to most antihistaminic substances, yet having a duration of activity extending at least 12 hours.

During the course of an antihistaminic screening program in these labortories, more than 50 compounds of the general series 1,2-diaryl-4-amino-2-butene were studied. These have been prepared by Drs. Mills and Rohrmann of the Organic Chemistry Division. One compound in particular was found to be singularly outstanding with regard to activity and length of action. This substance was the diphosphate salt of 1-*p*-chlorophenyl-2-phenyl-4-pyrrolidino-2-butene. This report deals with the pharmacology and toxicity of this compound.

General properties. The diphosphate salt of 'Pyronil' is a white crystalline substance with a melting point of 129.5-130°C (corrected). It is soluble in warm water to the extent of 10%. Most of the experimental data were obtained with the diphosphate salt, however, some experiments were performed with the hydrochloride. The structural formula of the drug appears below:



It will be noted that it bears a *p*-substituted chlorine in one of the benzene rings. In this respect it resembles structurally 2 other long-acting antihistaminic agents, namely, chlorcyclizine and chlorprophenpyridamine.

Antihistaminic properties. Isolated guinea pig ileum. An automatic device similar to one described by Gaddum(1) was employed to evaluate the relative activity of 'Pyronil.' This apparatus automatically stimulated at regular intervals a small segment of guinea pig ileum in a constant temperature bath containing histamine 0.5 μg per ml in Tyrode's solution. The contractile responses were recorded by the usual method on a kymograph. 'Pyronil', when applied to the muscle strip in sufficient quantities 2.4 minutes before the regular histamine stimulus, depressed the next few succeeding responses. The extent of depression depended upon the dose. A concentration of 'Pyronil' in the muscle bath of 0.005-0.01 μg per ml inhibited the histamine response about 50%. A comparison of the drug with diphenhydramine under these conditions indicated that the new antihistaminic exceeded diphenhydramine in activity 14.0 ± 1.65 times. The precision of this ratio was determined by the method of Loewe(2). The persistent activity of 'Pyronil' in contrast to diphenhydramine made itself apparent in these tests. After a single treatment with the new drug, many repeated treatments with histamine were necessary before the strip recovered its normal response.

Protection of normal guinea pigs in an aerosol of histamine. Antihistaminic activity in normal guinea pigs was studied with a modification of a method described by Halpern (3). A histamine aerosol was generated at a constant rate from 1.0% solution of histamine acid phosphate. This was passed through a chamber wherein 3 animals at a time could be observed. Only those animals showing definite signs of asphyxia within 3 minutes were chosen for test. Since the animals were not exposed long enough to be killed, they could be used repeatedly. The aerosol generator was pat-

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* 'Pyronil' is a trade mark identifying Eli Lilly and Co. brand of Pyrrobutamine.

TABLE I. Antihistaminic Activity in Guinea Pigs Subjected to a Histamine Aerosol.

Drug	Route of administration	No. of animals used	$ED_{50} \pm$ S.E., mg/kg	Minimal ED_{100}, mg/kg	$ET_{50} \pm$ S.E., hr
'Pyronil'	Oral	47	.79 ± .125	2.75	13.3 ± .55
	Subcut.	35	.059 ± .007	.2	4.8 ± .54
	Intramusc.	68	.063 ± .009	.14	5.3 ± .35
'Pyronil' hydrochloride salt	Oral	126	.78 ± .096		
'Histadyl'		166	5.5 ± .99	36.5	3.5 ± .57
Chlorcyclizine HCl		64	2.31 ± .53	10	6.5 ± .22
Tripelennamine "		37	3.23 ± 1.26	20	4.1 ± .51
Diphenhydramine "		46	16.48 ± 2.2	70	8.1 ± .44

terned after a design published by Dautrebande(4). The animal weight ranged between 175 and 350 g. The drug 'Pyronil' was then administered orally, subcutaneously or intramuscularly to the histamine sensitive animals. Thirty to 60 minutes later, and hourly thereafter, the animals were tested in the chamber for their ability to withstand the histamine. An animal was considered to be protected if it remained in the aerosol for 3 minutes without showing any signs of asphyxia. The number of animals per group varied from 5 to 10, and the dosage schedule was altered sufficiently so that it ranged from no protection to complete protection of animals in a group. From the percentage of the animals protected in a group and the dose administered, a median protective dose or ED_{50} was calculated by the method of Bliss (5). These data for drug 'Pyronil' are shown in Table I. In a similar manner the oral ED_{50}'s for diphenhydramine, 'Histadyl',[†] tripelennamine and chlorcyclizine were estimated. They are listed in Table I. An estimate of the duration of action of this antihistaminic drug was obtained from calculation of the ET_{50} for the minimal dose which protected all of the animals. The ET_{50} represents the median time for recovery of histamine sensitivity of a group of animals. This value was calculated by the graphic method of Litchfield(6). The ET_{50}'s for 'Pyronil' as well as the other antihistaminics mentioned are listed in the last column of Table I. It will be observed that 'Pyronil' was more potent and its effect more persistent than any of the other antihistaminics with which it was compared.

Antihistaminic activity on anesthetized animals. The intravenous injection of 1.0 mg per kg of 'Pyronil' into an anesthetized cat abolished the vasodepressor response of 0.8 μg of histamine similarly administered. The vascular reactions of 0.2 μg dose of methacholine iodide and 5 μg of epinephrine hydrochloride were unaltered by the antihistaminic.

Other pharmacological effects. Several types of smooth muscle tissue suspended in Tyrode's solution by the usual Magnus method were used to study the reactions of the antihistaminic. The motility of an isolated segment of rabbit ileum was depressed by the addition of this drug, 2.5-5.0 μg per ml final concentration, in the bath fluid. The movements of an isolated portion of a virgin rabbit's uterine horn were depressed when the concentration of the antihistaminic drug in the bath fluid reached 25 μg per ml. On the isolated guinea pig ileum this compound inhibited the contractions of methacholine iodide 0.5 μg per ml about 1/50 to 1/100 as effectively as atropine sulfate. The intravenous injection of doses of 1.6 to 6.4 mg per kg of 'Pyronil' (hydrochloride) into an anesthetized dog depressed the mean blood pressure for several minutes. Respiratory rate increased during the hypotension. In a second dog, both the increased secretion of saliva and the spasm of a section of the duodenum provoked by the intravenous injection of small doses of methacholine iodide 10 μg per kg were only very slightly diminished by doses of 'Pyronil' (hydrochloride salt), 1.0-3.0 mg per kg given by vein. By contrast, a small

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[†] 'Histadyl' is a trade mark identifying Eli Lilly and Co. brand of Thenylpyramine.

TABLE II. Acute Toxicity Studies—'Pyronil' and 'Histadyl.'

Drug	Animal species	Route of administration	No. of animals used	LD_{50} ± S.E., mg/kg
'Pyronil'	Mouse	Intrav.	150	53.53 ± 1.61
	"	Subcut.	150	1270 ± 156
	"	Intramusc.	21	836.8 ± 95
	"	Oral	48	1116 ± 73
	Guinea pig	Subcut.	37	1241 ± 165
	"	Intramusc.	29	625.6 ± 41.9
	"	Oral	24	992.6 ± 107
'Histadyl'	Mouse	Intrav.	80	19.85 ± .69
	"	Oral	45	182.2 ± 12.8
	Guinea pig	"	20	374.9 ± 34.5

dose of an anticholinergic agent completely nullified these responses. Apparently the drug has little anticholinergic activity.

Winter(7) has shown that there appears to be some correlation between clinical sedation observed with some antihistaminics and their ability to prolong barbiturate anesthesia in mice. The new compound was therefore compared with 'Histadyl' and diphenhydramine by Winter's method as regards its ability to prolong the sleeping times of mice lightly anesthetized with hexobarbital sodium. The dose level of the antihistaminics was 10 mg per kg by subcutaneous injection, whereas the barbiturate was given intraperitoneally, 100 mg per kg. The results of this comparison indicated that the new antihistaminic was more effective than 'Histadyl' but less potent than diphenhydramine. At this dose range, 'Histadyl' did not significantly prolong sleeping time.

One experimental condition which may be produced in certain animals and which closely resembles some of the clinical entities observed in allergy is the production of a high degree of sensitivity to some foreign protein. In guinea pigs it is relatively simple to sensitize the animal to some protein such as egg white and to bring on a condition of anaphylactic shock by subsequent injection of the same antigen at a later date. In order to demonstrate the effectiveness of the new antihistaminic in anaphylactic shock, 16 guinea pigs were sensitized by the intraperitoneal injection of 0.5 mg of crystalline egg white. Twenty-one days later the animals were divided into 2 groups of 8 animals each. One group served as a control while the animals in the other group were treated one hour in advance with 20 mg per kg by mouth of antihistaminic. All animals were then challenged with a shocking dose of the antigen by vein. Only 2 of the control animals survived, whereas 7 of the 8 treated animals were able to withstand the shock.

Toxicity studies. 1) *Acute toxicity.* The toxicity of 'Pyronil' was studied in several species and by various routes of administration. The median lethal doses as calculated by the method of Bliss in mice and guinea pigs are compiled in Table II. For the purpose of comparison, the LD_{50}'s of 'Histadyl' in the same species are also found in Table II. In the toxic dose range the compound produced convulsions followed by prostration and death, or recovery, usually within 72 hours irrespective of the mode of administration. In general it will be observed that 'Pyronil' in these 2 species was less toxic than 'Histadyl'. A comparison of the effective and the lethal doses of the new drug in guinea pigs by various routes of administration reveals that in these animals it has a high margin of safety. Antihistaminic substances appear to be more toxic in rabbits than in some of the other rodents (8). 'Pyronil' injected by vein in rabbits in toxic doses caused convulsions with collapse and death apparently due to respiratory failure. Although the number of animals was too small to determine a median lethal dose accurately, it appeared to be in the range of 15 to 20 mg per kg since one of 5 animals died on 15 mg per kg, while 4 of 5 animals died on the 20 mg per kg dose. Animals receiving doses of 8, 10, and 12 mg per kg all survived the treatment and did not appear to be ill during a 72-hour observation period. These rabbits were sacrificed and their organs examined after microscopic section. One of the 5 animals receiving 8 mg per kg had a few red blood cells in the renal collecting tubules. The other 4 were normal. Those animals receiving 10 mg per kg all had a small amount of blood in the renal collecting tubules. Thrombi appeared in the pulmonary arteries of one animal and endothelial proliferation was observed in the pulmonary arteries of another. A dose of 12 mg per kg caused a small amount of blood to appear in the renal collecting tubules of 2 rabbits, while thrombi were found in the pulmonary arteries of 3.

2) *Chronic toxicity in rats.* Four groups of 5 female rats each were fed diets containing various concentrations of the drug for 4 weeks using the method previously published(9). An additional group of 5 rats was fed normal rat ration as a control. The concentrations used, daily food and drug intake and effect on body weight follow:

% drug in diet	Avg daily food intake, g	Avg daily drug intake, mg	Avg body wt change, g
.05	13.01	6.51	+36
.10	12.99	12.99	+40
.20	11.88	23.76	+20.8
.50	9.43	47.15	—13.8
Control	13.88	—	+38.4

Hemoglobin levels were determined and erythrocyte, leukocyte, and differential counts were made on the animals receiving 0.2 and 0.5% diets prior to and at intervals during the 4-week treatment period. One rat on the 0.05% diet died after 10 days with an infection characterized by necrosis of the liver and spleen. Another rat on the 0.1% diet died after 9 days. Pulmonary edema and atrophy of the thymus were present. All others survived, were sacrificed and submitted for necropsy. Gross and microscopic studies of the heart, lungs, liver, spleen, intestines, kidney, thymus, thyroid, and adrenals, as well as the hematological studies showed no abnormalities attributable to the treatment.

3) *Chronic toxicity in dogs.* Three groups of 3 female dogs each were given daily doses of 'Pyronil' by mouth. The dose levels were 5, 10, and 15 mg per kg. Hemoglobin and non-protein nitrogen levels as well as erythrocyte, leukocyte, and differential counts were made weekly. Urinary tests for sugar and albumin were made at the same time. All animals survived a 4-week treatment period and were then sacrificed. Gross and microscopic examination of the heart, lungs, liver, spleen, stomach, intestines, kidneys, pancreas, thyroid, and adrenal glands were made.

With the exception of one animal there were no appreciable body weight changes observed during the 4-week period. This dog exhibited a considerable loss in weight, hemoglobin, and red cells, and on sacrifice was found to have a myocardial lesion. Since the changes in this animal were unique, they were not considered to be attributable to the drug. Among the other 8 dogs there were slight fluctuations in hemoglobin and red cells with over-all loss. These last observations might have been associated with the presence of erythrocytes found in the collecting tubules at the apices of the renal pyramids of the kidneys of 3 of the 9 dogs. One animal on the 5 mg per kg dose and 2 dogs on the 15 mg per kg dose showed this effect. The number of red cells was small and they were present in only a small number of tubules. That this finding is of no great significance is borne out by the fact that the benzidine test of urine obtained by needle aspiration of the bladder at necropsy was positive in only one instance. The lack of change in non-protein nitrogen levels in blood and the normal urinary findings were proof of the absence of any injury to kidney tissue. Although the leukocyte counts showed the usual fluctuations, they were never significantly depressed. All other tissues examined in these animals were normal.

Conclusions. 1. 'Pyronil', a new histamine antagonist, was found to possess an antihistaminic activity of remarkable degree and duration in 2 different test preparations. Its other pharmacological effects, aside from its ability to protect guinea pigs from anaphylactic shock, were unimportant. 2. In mice and guinea pigs 'Pyronil' had a relatively low toxicity when administered orally, subcutaneously or intramuscularly. In mice by vein it was moderately toxic although apparently less toxic than many of the other antihistaminics in mice as judged from published lethal doses in the literature(8). In guinea pigs the ratio of toxic to therapeutic doses indicated a high margin of safety. In rabbits this compound had the same toxic dosage level as other antihistaminics. 3. Rats fed diets containing as high as 0.1% of the drug in the diet for 4 weeks gained weight comparable to the weight of control rats fed normal ration. Higher concentrations impaired growth but produced no visceral damage as shown by hematological and pathological studies. 4. Dogs given large daily doses of 'Pyronil' for 4 weeks, aside from the presence of occasional red cells in the collecting tubules of the kidney, showed no significant pathological changes attributable to the drug.

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Some Factors Involved in the Fibrogenesis of Collagen *in vitro*.[*] (19657)

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In solutions of collagen (ichthyocol) obtained by filtration of dilute acetic acid extracts of carp swim bladder tunic, the collagen is dispersed as extremely thin filaments, or highly asymmetric particles, less than 50 A in width, rather than as cross-striated fibrils characteristic of native collagen(1). By suitable adjustment of ionic strength (usually with NaCl, though many other salts are effective) and of pH, cross-striated fibrils typical of native collagen as seen in the electron microscope, are precipitated(1-5). The authors previously reported(2) that reconstitution of collagen-type fibrils could be effected by addition to the acid filtrate of serum acid glycoprotein(6,7) in a concentration of about 10^{-9} M (0.001%) followed by dialysis against water. If the concentration of glycoprotein (GP) is higher (ca 10^{-7} M, or 0.05%), the reconstituted fibrils have the long-spacing (LS) pattern, with an axial period of about 2100 A, previously observed in the "procollagen" obtained by dialysis of acid citrate extracts of connective tissue(8). Because GP is so effective in reconstitution, it was suggested(2) that GP, or a component thereof, may be involved in the fibrogenesis of collagen. This effectiveness in reconstituting collagen-type fibrils in the absence of inorganic ions does not, in itself, constitute evidence that GP plays any role in the natural process of collagen fibrogenesis. However, analytical evidence, too scanty to warrant description at the time, supported the view that certain of the components of glycoproteins and polysaccharides are present in the precipitated cross-striated fibrils, whether of the collagen- or LS-type.

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Further experimentation proceeded along 2 lines: 1) extensive chemical analysis of collagenous tissue sufficiently purified to remove the ground substance and other materials not firmly bound within the native fibrils; 2) extensive testing of the uniqueness of GP in its ability to reconstitute collagen- and LS-type fibrils from acid solutions of ichthyocol. This paper concerns the second aspect. The results support the view that the potentiality for reconstitution resides in the extracted fibrous material itself; various substances, including some which have no obvious relationship to glycoproteins or mucopolysaccharides, were found capable of inducing reconstitution.

Experimental. Ichthyocol solutions are prepared by allowing 0.6-0.8 g of scissor-minced, water-washed carp swim bladder tunics to stand in 100 ml of 0.05% acetic acid, pH 3.5, for 24 hours at 0°C, with occasional stirring, avoiding extensive comminution of the tunics and continuous stirring during extraction. The viscous suspension is passed, consecutively, through bolting cloth, coarse, medium and fine sintered glass filters. The final filtrate is clear and viscous, containing about 0.1% protein. It may be stored in the cold for several months. All filtrates are examined carefully with the electron microscope for intact fibrils. A rare striated fibril which slips through the filters may be tolerated. One ml of solution containing the substance to be tested (dissolved in acetic acid, pH 3.5), in graded concentrations ranging from 0.002% to 5%, was mixed with one ml of ichthyocol filtrate. Any precipitate which appeared within 30 minutes was examined in the electron microscope. All preparations were then dialyzed against distilled water at room temperature without removing any precipitates which may have formed. After dialyzing 12 to 18 hours, the preparations were reexamined.

The test substances may be grouped in 3 categories depending upon the type of response evoked.

1. *Reconstitution of collagen- and LS-type fibrils.* In this group are included: Serum acid glycoprotein(6,7), thrombin (Lilly, Seegers), papain, *Cl. histolyticum* proteinase (MacLennan), and ACTH (Armour, Parke-Davis). Upon mixing with ichthyocol filtrate no precipitate was produced. After dialysis against water, heavy precipitates appeared which, with the exception of those from the ACTH mixture, contained well-formed collagen- and LS-type fibrils. Only collagen-type fibrils occurred in the ACTH precipitates. Seegers'(9) data suggest that his thrombin preparations may contain a constituent having electrophoretic properties similar to those of GP. Solutions of the above substances may be heated at 95°C for 30 minutes, followed by treatment with 20% trichloracetic acid, without altering the capacity to reconstitute striated fibrils; if anything, the perfection of structure thus produced was greater than with the untreated substances. In the above experiments, when precipitation occurred on treatment with trichloracetic acid, the solutions were clarified by centrifugation, the supernates dialyzed and aliquots used for the experiment. GP is not precipitated by boiling or subsequent treatment with 20% trichloracetic acid.

2. *Reconstitution of unstriated and LS-type fibrils:* In this group are included: Sodium and potassium hyaluronates, calcium and potassium chondroitin sulfates (K. Meyer, R. W. Jeanloz), and heparin (Lilly). Immediate heavy, clumped, fibrous precipitates appeared on addition of the acid mucopolysaccharide solutions to the collagen filtrate in the absence of salt. Electron microscope examination revealed fibrils of a variety of sizes, anastomosing and forming sheets, but exhibiting no cross-striations. Dialysis against water did not alter this picture. One preparation of calcium chondroitin sulfate from cartilage (K. Meyer) produced some typical LS fibrils on addition to filtrate, but no collagen. Although these polysaccharides reconstitute chiefly unstriated fibrils, as reported previously(2), it was found that LS- but not collagen-type fibrils were produced when the reaction was carried out in the presence of salt (NaCl, $\mu = 0.17$). Under these conditions, pronounced opalescence with some fibrous precipitate appeared immediately. Examination revealed large masses of unstriated fibrils plus smaller clumps of typical LS forms. No collagen-type fibrils were seen. Subsequent

dialysis of the preparation against water produced no apparent change. These structures were observed over the entire range of concentrations of mucopolysaccharides tested.

3. *No reconstitution.* In this group are included: Crystallized bovine serum albumin, fibrinogen (Seegers), gliadin, and protamine sulfate. No precipitation or opalescence appeared upon mixing solutions of these substances with ichthyocol filtrates or after dialysis against water. Electron microscope examination revealed no fibrils.

As controls for all of the above experiments, ichthyocol filtrates were dialyzed against water. In all cases clear gels were produced in which very thin, unstriated fibrils and filaments were observed.

It is important to note that salt-reconstituted ichthyocol contains all the materials necessary for the production of LS- as well as collagen-type fibrils. This was demonstrated as follows: An ichthyocol solution was reconstituted by dialysis against 1% NaCl, pH 6-7. The saline-washed precipitate was extracted with citrate buffer ($\mu = 0.2$, pH 3.8) and the extract dialyzed against water. A fibrous precipitate formed which contained both collagen- and LS-type fibrils. Apparently the salt-reconstituted collagen contains something which is required for the production of LS-type fibrils. The fact that hexosamine and tyrosine have been demonstrated to be present in the salt precipitate may be significant in this connection.

Discussion. The above experimental results suggest that, in seeking to understand the mechanism of *in vitro* collagen fibrogenesis, it will be profitable to focus attention on the fibrous system extracted from the connective tissue rather than upon the substances which, under various conditions, may be able to induce the reconstitution of collagen- or LS-type fibrils. From the present results it is obvious that a variety of inorganic and organic compounds, under specified conditions of ionic strength and pH, may be able to cause the fibrous material to aggregate into fibrils having specific structural patterns. Such behavior requires the assumption that the test substances interact with one or another aspect of the structural organization inherently present in the extracted fibrous material.

Although the fibrous protein is doubtless responsible for the major structural properties of the extracted material as well as those of the native fibrils, it has long been known that a carbohydrate component is also present in skin collagen in a concentration of about 1% (10-12). The present authors have been able to show by analyses of purified cowhide collagen, to be described in detail elsewhere, that there is a carbohydrate-containing fraction in the collagen fibrils. This fraction includes hexoses (glucose, galactose, mannose), and hexosamine as well as tyrosine and tryptophan. Although these substances are constituents of the serum acid glycoprotein molecule, it has not yet been possible to isolate a glycoprotein from collagen fibrils demonstrably free of ground substance.

We have been able to demonstrate the presence of hexose, hexosamine and tyrosine in the fibrils reconstituted by salts from acid solutions of ichthyocol as well as in ichthyocol purified by thrice repeated cathodal electrodeposition (13) from acid solutions.

To what extent the carbohydrate-containing fraction is involved in the structural organization and fibrogenesis of collagen remains to be demonstrated.

Summary. 1. A variety of organic substances derived from animal and plant sources can induce the precipitation of collagen- and long-spacing-type fibrils from acid extracts of ichthyocol in a manner similar to that of serum glycoprotein. These fibril-precipitating agents appear to be thermostable and are not altered by subsequent exposure to 20% trichloracetic acid. 2. Hyaluronate, chondroitin sulfate and heparin produce fibrous precipitates directly on addition to ichthyocol filtrates. At low ionic strength these fibrils are unstriated. At ionic strengths of 0.1 to 0.2 (NaCl) some long-spacing-type fibrils are formed but collagen-type fibrils have not been observed. 3. The components necessary for the formation of cross-striated fibrils appear to be contained in the fibrous material extracted from the connective tissue; the action of the precipitating substances seems to be relatively non-specific under the conditions of these experiments.

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Reactions of the Vessels of the Rat Kidney after Experimental Occlusion of the Renal Artery for Various Periods. (19658)

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The kidney of the rat is well adapted to the study *in vivo* of gross vascular changes. The renal vessels of the rat appear to be especially sensitive to vasoconstrictor influences, and the vessels on the surface of the kidney are too few to interfere with color changes resulting from changes in the caliber of, and in the blood flow through, vessels of the superficial cortex. Therefore, we have used the rat in making gross observations concerning the reactivity of renal cortical vessels after varying periods of occlusion of the renal artery.

We previously observed that renal vasoconstriction appears to be limited to the vessels of the cortex(1,2). When the renal artery was suddenly completely occluded, the entire cortex of the kidney blanched severely, provided the vessels had not been manipulated previously. The cortical vessels evidently constricted briefly and squeezed the blood from the cortex. However, study of cut sections showed that the medulla retained its normal red color and its vessels remained filled with erythrocytes.

When the occlusion of the renal artery was released after only 5 to 15 seconds, the rat kidney appeared hyperemic for the next 10 to 30 seconds, as indicated by the bright pink color and the fullness of all vessels on the surface of the kidney when magnified and observed through a dissecting microscope. The kidney rapidly regained its normal color and appearance after this transient occlusion. Evidently, a brief period of hyperemia followed the brief period of ischemia.

When the occlusion of the renal artery was maintained for approximately one minute, after release of the occlusion the surface of the kidney appeared dark red or blue for 3 to 7 minutes, after which the normal color returned. If only one branch of the renal artery was occluded for a similar period, that portion of the kidney supplied by this vessel showed the congested appearance after release of the occlusion. The congested appearance of the portion of the kidney supplied by the vessel previously occluded could be contrasted with the normal appearance of the remainder of the kidney. After an intravenous injection of India ink, the region supplied by the vessel

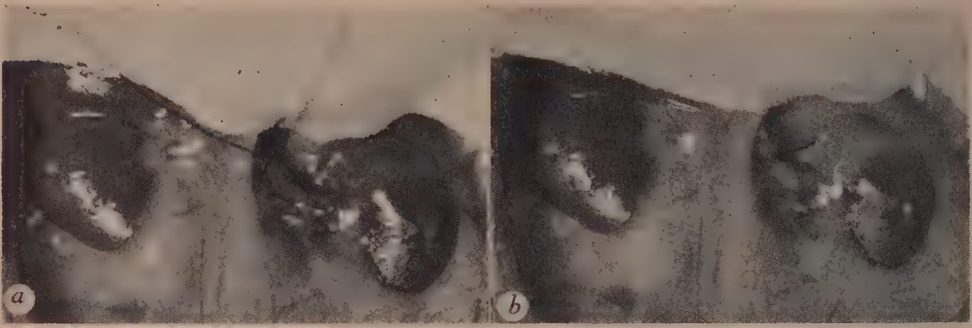

FIG. 1. Failure of congested renal vessels to constrict again shortly after previous occlusion of the supplying artery: *a*, branch of the renal artery supplying the lower half of the kidney on the right was occluded for 40 sec, during which this area of the kidney blanched; after release of the occlusion of the supplying artery, the involved half of the kidney became congested and dark in color, as shown; *b*, same preparation as shown in *a*: the main renal artery to the kidney, shown on the right, was tied off immediately after the picture shown in *a* was taken; the upper half of the kidney then blanched, as shown, but the lower half remained congested, indicating that vessels in this half of the kidney failed to constrict again.

previously occluded filled with ink more slowly than did the unaffected renal tissue, but became darker in color and the ink left it at a slower rate than that at which it left the normal renal tissue. Thus, it appeared that blood was flowing at a slower rate in the dilated vessels in the region of the kidney, the artery to which had been previously occluded for a brief period.

After the renal artery was released after a brief clamping, reclamping after a few seconds would not cause the kidney to again blanch so markedly. This was clearly demonstrated by first clamping one branch of the renal artery for a minute (that region of the kidney supplied by the vessels blanching during this time), then releasing this clamp for 5 or 10 seconds, and then occluding the main renal artery. After the main renal artery was clamped, the region of the kidney supplied by the branch of the artery first occluded remained dark, while the remainder of the kidney supplied by arterial branches not previously occluded blanched markedly (Fig. 1). Thus, after a brief period of severe ischemia, the vessels of the kidney lose the ability to constrict. However, when the interval between repeated brief clampings of the renal artery was more than a minute, the vessels regained the ability to constrict with each occlusion of the renal artery.

When the renal artery was occluded for a period of 20 to 60 minutes, the recovery of blood flow through the kidney at first appeared only in certain regions in a haphazard pattern. Gradually the flow returned to all regions of the kidney within 10 to 15 minutes, and after that the kidney regained its normal color. When the renal artery was occluded for 2 or 3 seconds at intervals of 5 to 10 seconds, the kidney became dark and greatly congested. The entire cut surface of such a kidney also appeared congested. These changes occur independent of renal innervation; that is, they occur in the denervated kidney as well as in the innervated organ.

Summary. 1. Brief cortical vasoconstriction of the kidney followed occlusion of a renal artery not previously manipulated. When occlusion of the artery was maintained only a few seconds, an equally brief period of renal hyperemia followed. 2. More prolonged occlusion produced a short period of renal vasodilatation and congestion followed release of the occlusion. During this period of dilatation, the vessels did not respond by vasoconstriction to repeated occlusion of the renal artery.

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Infra-red Spectroscopy of Brain Tissue. A Lipid Fraction in Normal and Irradiated Adult and Fetal Rats.* (19659)

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Previous studies have demonstrated that brain tissue shows characteristic but species unspecific infrared absorption spectra. The present paper reports: 1. Differences in qualitative and quantitative spectroscopic findings in the brain of adult and developing rats. 2. Tentative assignment of brain tissue spectra. 3. Spectroscopic studies of an effect of ionizing irradiation on developing and adult brain.

Material and methods. Female albino rats of about 200 g were used for the experiments in adult brain and pregnant rats of between 2-3 weeks gestation for the studies in fetal brain. The animals were killed by decapitation. The brain was removed quickly, and frozen and dried sections through brain stem, hypothalamus, midbrain, cerebellum and forebrain of adults and through the whole brain of fetuses were prepared for spectroscopy. Histologic studies were made from sections cut from the block remaining after sections for spectroscopy were taken. The technic of preparation of sections and infrared spectroscopy of tissues was essentially the same as described previously(1). The irradiation experiments comprised administering total body irradiation from 500-800 r in non-pregnant and from 150-400 r in pregnant animals. Adult non-pregnant animals were studied one or 2 days after the irradiation. Fetuses were removed for spectroscopic and pathologic studies 24 hours after exposure of the mothers to x-rays. In a few instances studies were carried out in animals born from 1-2 days after irradiation of the mothers. Results obtained in those animals were compared with controls of the same age. Irradiation was delivered from a Villard Westinghouse machine at 200 KW, 20 milliamperes with 1 mm aluminum and ½ mm copper filters. The rats were irradiated in thin-walled plastic or glass containers. The target distance was 52 cm. The x-ray dosage was given in a single exposure at the rate of 50 r per minute. The amount of irradiation administered under those circumstances was checked at intervals with a Victoreen symbol ionization chamber placed in the empty containers. According to those measurements, variations of selected dosage were not more than a few r from experiment to experiment.

Results of qualitative infrared spectroscopy of adult and developing brain tissue. Two different types of absorption bands were found within the different wavelength regions. 1. In the high frequency region, below 8.1 μ adult animal, fetus and newborn alike showed a number of more or less strong bands at locations almost similar to known vibrations (3.04 μ NH stretching, 3.4 μ CH bending, 6 μ CO stretching, the deformation vibration of NH at 6.44 μ, and the deformation vibration of CH at 6.88 μ found in proteins and polypeptides (Sutherland)(2). Additional weak or medium strong bands occurred at 7.20 μ (methyl vibration), and at 7.70 μ, 8.10 μ, and 8.53 μ (unassigned). 2. In the low frequency region, *e.g.*, between 8.1 μ and 11 μ, the spectra of adult and fetal brain tissue showed significant differences. In adult rats rather strong bands at about 9.26 μ and weaker bands at about 10.28 μ were found. In fetuses and newborn, weaker bands occurred regularly at 9.23 μ, 9.56-9.65 μ (a shoulder like absorption band) and at 10.30 μ.

Fig. 1 and 2 show transmittancy curves which were obtained from sections of about the same thickness (50 μ) cut through adult and fetal brain. The great differences between the transmittancies of adult and fetal brain tissue are apparent. The transmittancy curve

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† Acknowledgement is gratefully made for the cooperation and assistance of Dr. B. P. Widmann and members of the staff of the Division of Radiology

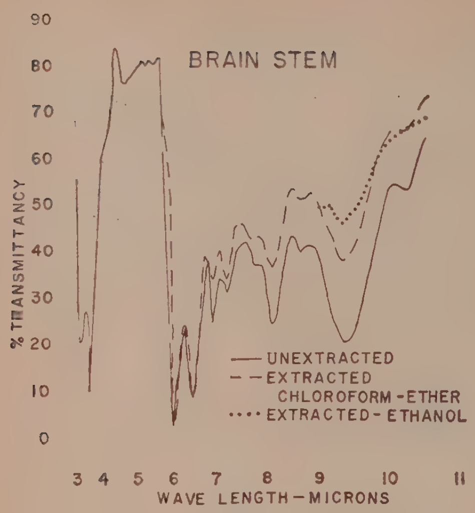

FIG. 1.

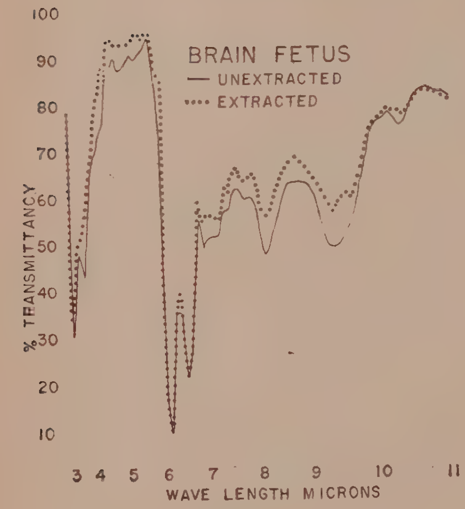

FIG. 2.

of the fetal brain (solid line in Fig. 2) closely resembles the curve of extracted adult brain tissue (interrupted and dotted line, Fig. 1).

Tentative assignments of brain tissue spectra. Lipids are contained in neural tissue in so high concentration that it was reasonable to investigate their role in producing the characteristic spectra of brain tissue. For this purpose infrared spectra were recorded from brain tissue sections before and after extraction with various lipid solvents, from evaporated films obtained from some of those extracts, and from pure compounds related to lipid metabolism. The mounted frozen and dried brain sections were extracted with mixtures of solvents such as chloroform:ether (1:1), or petroleum ether:methanol (9:1), or simple solvents (ethanol, benzene, and petroleum ether) at 4°C. The time of extraction varied from 15 to 50 hours until spectroscopic examination revealed no further effect of the extraction. The effect of chloroform:ether and ethanol extractions, which alone will be discussed here are shown in Fig. 1, and can be briefly summarized as follows: Ether:chloroform extraction greatly reduces the characteristic absorption bands of brain tissue in the 9-11 μ region. This extraction does not affect the protein amide vibrations such as the deformation vibration of NH at 6.44 μ, in the high frequency region. Ethanol extraction has even greater effect in reducing the characteristic absorption spectra and in addition sometimes measurably decreases the strength of the amide vibrations (deformation vibration of NH at 6.44 μ). The chloroform:ether extracts remaining after extraction of the brain sections were concentrated in vacuum and finally evaporated in specially designed silver chloride wells of about 15 mm diameter and 1/10 mm depth. The films, thus obtained, showed spectra which were related to the characteristic bands of brain tissue and in addition a number of bands which were not visible in the spectrum of the tissue itself, when overshadowed by absorptions of the background.

Fig. 3 shows a transmittancy curve of one of those films. Films obtained from numerous brain sections gave, except for slight variations, similar spectra. The general pattern of these spectra comprises, a strong band at about 5.70 μ, a strong triplet at about 7.8 μ, 8 μ, and 8.30 μ, a weak band at 8.5 μ, strong bands at about 8.80 μ and 9.20 μ, and weaker and broader bands near 9.80 μ and 10.30 μ. These spectra show some very characteristic groups of bands which closely resemble those of glycerides of long chain saturated and unsaturated fatty acids (Shreve *et al.*(3)) and in part the few spectra of cephalins studied by Baer(4). The strong band at 5.7 μ indicates

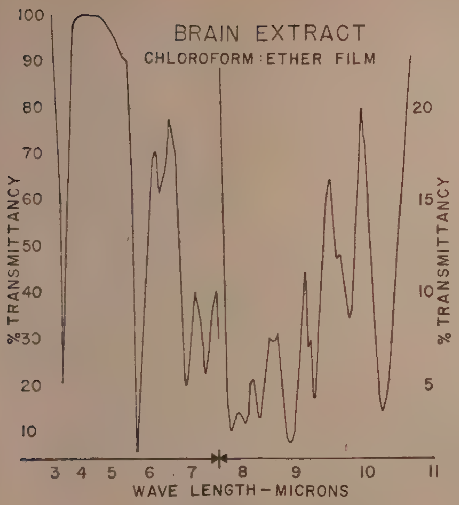

FIG. 3.

the presence of esters and the triplet below 9 μ corresponds to vibrations of fatty acid glycerides. The strong band near 9.20 μ, and the weak bands at about 9.80 μ and 10.30 μ could not be assigned so easily. It was assumed tentatively that the strong band at 9.20 μ might be produced by C-O or C-O-P vibrations contained in phospholipids and glycolipids, and that the weak band at about 10.30 μ would be, at least in part, produced by the specific vibration (at 10.36 μ) of the trans double bond found in trans unsaturated compounds and their esters. The band at about 9.80 μ remains unassigned. In reviewing the results of our studies of brain extracts, it should be stated that some of the assignments must be considered with caution. The fact that constant and characteristic spectra of a brain lipid fraction can be obtained from such small amounts of tissue suggests that spectroscopic examination of brain extracts are valuable for investigation of lipids in the brain.

Many pure compounds were studied to confirm the tentative assignments. These included unsaturated and saturated fatty acids, phospholipids (cephalin, L-α-cephalin, lecithin free of cephalin, and sphingomyelin), glycolipids (cerebrosides, kerasine, and phrenosine), sphingosine sulfate, sodium glycerophosphate, cholesterol, and cholesterol acetate. Most substances were obtained from commercial sources, L-α-cephalin was kindly supplied by Dr. E. Baer of Toronto and sphingomyelin by Dr. S. Tannhauser of Boston. Study of the spectra of these compounds, which will be reported in detail elsewhere showed the correctness of some of our assumptions. All the glycolipids, phospholipids, as well as glycerophosphate showed rather strong bands at 9.20-9.30 μ, very close to the location of the strong characteristic band found in intact brain tissue and in the chloroform:ether extract. The spectra of glycolipids annd sphingomyelin, however, differed significantly from those of the brain extract in the 6-7 μ region.

In glycolipids and sphingomyelin the strong ester band of the extract at 5.7 μ was replaced by an amide C-O vibration at about 6.10 μ and an additional NH vibration at 6.46 μ which were never seen in any chloroform:ether extract. This finding, which could have been predicted from the amide structure of those compounds, proved that not glycolipids or sphingomyelin but amide-free phospholipids such as lecithins, cephalins and other lipid-like esters were the main constituents of the chloroform:ether fraction. Cholesterol esters are found in too small quantities in normal adult and developing brain tissue to make any considerable contribution to the absorption spectrum of the lipid fraction. Also the strongest bands of cholesterol esters and free cholesterol are located at distinctly higher wave lengths than the strong bands of intact brain tissue and brain extracts in the 9-10 μ region. Measurements at 9.50 or 9.60 μ may include whatever amounts of free cholesterol (5) or cholesterol esters are present. Bands at about 10.30 μ, the location of the second characteristic band in brain tissue, were seen in many of the pure compounds but cannot be assigned more definitely at present. In reviewing the assignment work it can be stated that studies of more pure compounds and further fractionation of the chloroform:ether fraction may lead to final identification of the spectroscopically well defined amide-free lipid fraction.

Quantitative infrared spectroscopy. For quantitative estimation of the amide-free lipid fraction, spectra are recorded from mounted brain sections before and after extraction with

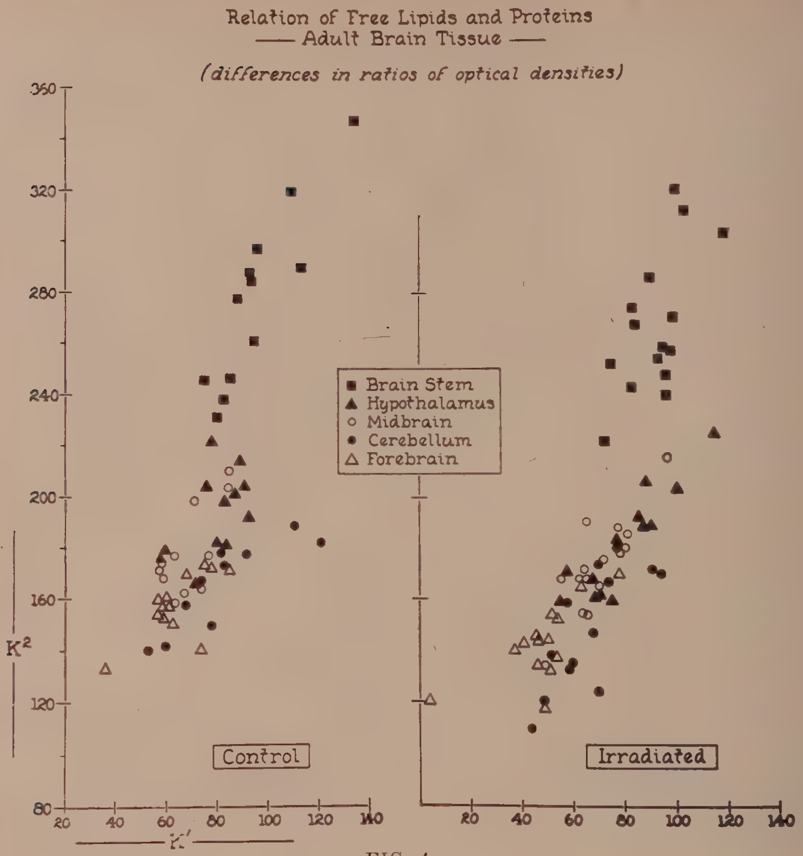

FIG. 4.

chloroform:ether. Ratios of optical densities between the characteristic bands of adult or fetal brain tissue in the 9-11 μ region and the amide bands at 6.44 μ are determined. These ratios of optical densities are independent of thickness of sections according to Beer's law. The differences of ratios of optical densities before and after the extraction are a relative measure of the relation of the amide-free lipids to the amides in the brain. Since the amount of amides from compounds other than proteins are relatively small, these differences of ratios of optical densities actually represent the ratio of the amide-free lipid fraction to proteins of the brain. Fig. 4 and 5 show the results in adult and fetal controls and the effect of ionizing irradiation. For the graphic presentation in Fig. 4 (adult brain), the differences of ratios were determined at 10.30 μ and 9.30 μ. The values obtained multiplied by 1000 were called K 1 and K 2 respectively. K 1 was plotted as abscissa and K 2 as ordinate. For the graph in Fig. 5, the differences of ratios of optical densities were measured at 10.30 μ, 9.50 μ, and 9.23 μ. The figures obtained multiplied by 100 were called K 1, K 2, and K 3, respectively. The K values were plotted as abscissa and the numbers of fetuses or newborn as ordinate.

Amide-free lipid fraction in adult and developing brain. Fig. 4 clearly demonstrates that a definite pattern of the ratios of amide-free lipids to proteins exists in adult brain with brain stem showing the highest and forebrain the lowest figures and hypothalamus, mid-

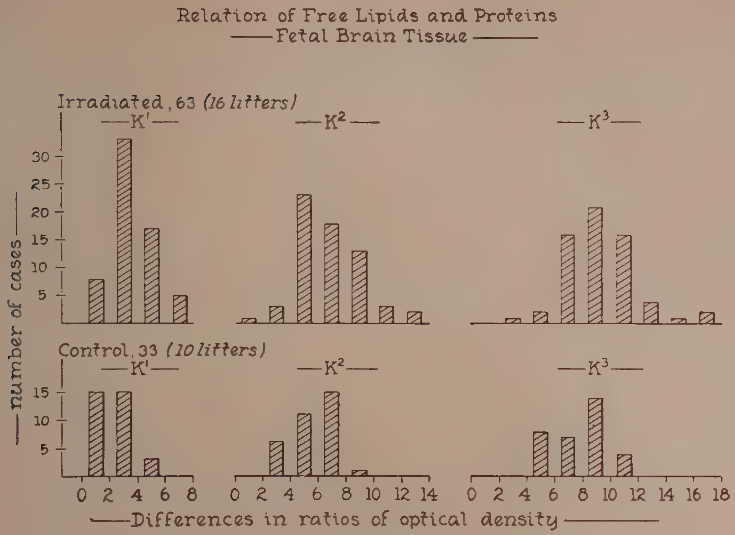

FIG. 5.

brain, and cerebellum between the 2 extremes. The differences between these ratios are so great and the change in the "protein amide" values in the dry tissue relatively so small that variations in the amounts of amide-free lipids rather than proteins are responsible for the pattern described. Comparison of the figures shown in this graph and in Fig. 5 indicates that the amount of amide-free lipids is significantly smaller in fetal brain. This finding explains in part the different appearance of spectra of adult and fetal brain (Fig. 1 and 2).

Effect of ionizing irradiation on the amide-free lipid fraction. As indicated in Fig. 4, ionizing irradiation with doses of from 500-800 r produces within 1-2 days a small but significant decrease of the amide-free lipid fraction. The effect is rather infrequent in animals subjected to irradiation with 500 r (half lethal dose) and increases, though not regularly, in frequency and intensity with higher dosage. The animals show no objective signs of abnormality of the central nervous system and routine histologic studies revealed no significant pathologic alterations. Total body irradiation of pregnant animals at 150-400 r causes an increase of the amide-free lipid to protein ratio in fetuses removed after one day and animals born 1-2 days after the irradiation of the mothers (Fig. 5). The effect occurred more frequently after irradiation at 300 r and above. Histologic studies revealed changes of the fetal brain similar to those described by Hicks(6). The ultimate effect upon the nervous system of irradiation *in utero* is the subject of further studies.

Discussion. The present studies demonstrate that the characteristic bands of the infrared spectrum of the brain are mainly produced by lipids. The amide-free lipid fraction can be completely extracted so easily from mounted brain sections (after 15 hours at 4°C) that it is very probable that this fraction is only loosely bonded or not bound to protein at all.

The variation of this lipid fraction in different parts of the brain suggests a possible relation of the concentration to the degree of neural activity since the largest amounts were found in areas of vital functions—the medulla—and the smallest in the forebrain, which in the rat has a relatively low functional development.

The concentration of the lipid fraction found spectroscopically in fetal brain may not be an entirely valid reflection of the total amount in developing brain tissue. Limitations of infrared spectroscopy employed in this investigation preclude quantitative studies of tissue sections below 3 x 3 mm. For this reason, the fetal brain was examined as a whole. As a result, the sections prepared for spectroscopy included mainly the immature forebrain structures which even in the adult state show the lowest concentration of the lipid fraction.

The increase of the fraction in the fetus following maternal irradiation may be related to the destructive effect of x-rays upon the germinal areas of the forebrain and reflect the results of a chemical breakdown of embryonic cell elements. The formation of peroxides in the skin lipids(7) of x-ray irradiated animals and x-ray irradiated linoleic acid *in vitro*(8), as it might affect the bonding of lipids to protein, may prove a fruitful avenue for further study of the mechanism of the results obtained.

It is expected that these results may contribute to the elucidation of the greater radiosensitivity of the developing brain recently demonstrated by Hicks and confirmed by our own histologic studies. It is also felt that infrared spectroscopy may be valuable for investigation of other complex problems involving lipids in the brain.

Summary. 1. Frozen and dried sections of brain tissue, brain extracts, and pure compounds related to lipid metabolism are studied by infrared spectroscopy. 2. The characteristic infrared absorption bands are tentatively assigned to certain lipid structures. 3. An amide-free lipid fraction is studied in adult and developing brain. 4. Adult and developing brain tissues show different infrared spectra. These differences are in part caused by different content of amide-free lipids. 5. X-ray irradiation decreases the amide-free lipid fraction in the adult and increases that fraction in developing brain.

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Correction of Anemia Following Hypophysectomy by Administration of Cobalt.[*] (19660)

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Polycythemia can be produced in normal rats by placing the animals in an environment of reduced oxygen tension(1) or by the injection of cobalt(2). Because cobalt is thought to produce polycythemia by interference with oxidative mechanisms(3,4) and because hypophysectomy interferes with the usual increase in red cells and hemoglobin in response to an environment of reduced oxygen tension(5,6) it was thought of interest to see if hypophysectomized rats could respond to cobalt with the production of an increased circulating red cell volume.[†]

It has been previously shown that 0.05 mg of cobalt and 0.1 mg of iron per 100 g of body weight per day for 60 days is sufficient to produce a 50% increase in the total circulating red cell volume of normal rats(7).

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[†] An abstract has recently appeared by Crafts(11) in which he reports that administration of cobalt repairs anemia of hypophysectomy.

TABLE I. Hypophysectomized Female Long-Evans Rats Uninjected or Injected Intraperitoneally for 53 Days with .07 mg Cobalt and .1 mg Iron per Day.

	Body wt, g	Hct., %	Hgb. conc., g/100 ml	TRCV, ml/100 g body wt	Total circ. Hgb., g/100 g body wt	% nucleated red cells in marrow
Normal controls	185	52	15.8	2.72	.827	
	199	50.7	15.6	2.16	.663	
	184	49	14.6	2.74	.816	
	187	39.6	12.2	1.89	.583	
Avg	189	47.8	14.6	2.38	.722	
Uninj. hyped	94	34.1	10.8	1.50	.477	31
	90	32	9.8	1.56	.479	37
	107	30.8	9.6	1.25	.392	39
Avg	97	32.3	10.1	1.44	.449	36
Cobalt inj. hyped	80	42.8	12.6	2.31	.679	29
	96	43.7	13.4	2.06	.633	34
	89	47.1	14.4	2.43	.739	33
Avg	88	44.5	13.5	2.27	.684	32

TRCV = total circulating red cell volume; hyped = hypophysectomized.

Methods. Five female rats of the Long-Evans strain, hypophysectomized 5 months previously at 28 days of age, were injected intraperitoneally with 0.07 mg cobalt as cobaltous sulfate and 0.1 mg of iron as ferric chloride per 100 g of body weight daily for 53 days. The ferric chloride was added so that sufficient iron would be available for the formation of hemoglobin. Four normal rats and 5 uninjected, hypophysectomized rats were kept as controls. Two of the injected and 2 of the uninjected animals died during the period of the experiment. At the end of the injection period the total circulating red cell volumes were determined by the Fe^{59} labeled cell dilution method. The labeled cells were obtained from a Long-Evans donor previously injected with Fe^{59}. The experimental animals were injected through the saphenous vein with 0.1 ml of donor blood containing 0.03 μc Fe^{59}. After allowing 6 minutes for the blood to mix, the abdomen was opened and a sample of blood was drawn from the vena cava into a heparinized syringe. A known volume of this blood was pipetted into a vial and its activity counted directly in a scintillation counter(8). The total blood volume was calculated from the fraction of the injected activity recovered in this sample. The hematocrits were determined in Wintrobe tubes. The total blood volume thus calculated multiplied by the hematocrit gave the total circulating red cell volume. The total circulating red cell volumes were then corrected for the body weights of the animals and the results are presented in terms of ml of red blood cells per 100 g body weight. The hemoglobin concentration was determined by the method of Turner(9). Marrow smears were made from the shaft of the femur and the percentage of nucleated red cells was obtained by counting 500 cells from each smear. At autopsy the pituitary site was examined under a binocular dissecting microscope to determine the completeness of hypophysectomy, and the adrenals, thyroid, and testes were weighed as additional evidence of the completeness of hypophysectomy.

Results. The results are summarized in Table I. The uninjected rats showed the anemia typical of hypophysectomy(10). The cobalt injected hypophysectomized rats showed a 58% increase in total red cell volume per 100 g body weight and a 52% increase in grams of circulating hemoglobin per 100 g body weight resulting in a normal total red cell volume and circulating hemoglobin. This 58% increase in total red cell volume is of the same magnitude as the 50% increase seen in normal rats injected with cobalt(7); however, in the latter case this results in the development of a polycythemia while in the former it merely restores the total red cell volume to a normal level. The percentage of nucleated red cells in the marrow smears was not increased. This was also true of normal rats injected with cobalt(7). This does not mean, however, that there was not an absolute increase in the number of red cell precursors in the marrow since sections of the marrow were not studied.

Conclusions. The anemia of hypophysectomy was repaired by the administration of cobalt. The increase in red cell volume was of the same magnitude as that reported in normal rats. Apparently the pituitary is necessary for a normal erythropoietic response to hypoxia but is not necessary to obtain a normal erythropoietic response from cobalt. In this way the mechanisms responsible in each case appear to differ.

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A Simple and Sensitive Histochemical Method for Calcium. (19661)

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A histochemical method for staining calcium should be both specific and sensitive. The commonly used Von Kossa silver nitrate stain(1) has great sensitivity but lacks specificity for calcium: its use depends upon the fact that it stains the anions usually in association with calcium, namely phosphate and carbonate. Some of the alizarin dyes were shown by Cameron(2) to have considerable specificity but lacked sensitivity, a defect that has not been remedied by a number of modifications(3-6). Because of low sensitivity this test was found to be inapplicable to the study of early pathological calcification during the period when only minute foci were present.

In conjunction with chemical and anatomical studies(7,8), investigations were undertaken aiming at a stain that was specific, sensitive, and simple. These studies have led to the development of an alizarin staining technic which is almost as sensitive(8) as the Von Kossa and yet which is specific for calcium under the usual conditions of normal and pathological calcification. With it, studies have shown very early intracellular calcification at a time when the Von Kossa test was completely negative, thereby revealing the inadequacy of the latter as an index of the calcium present. Calcium deposits have been demonstrable in tissues from 12-48 hours before microanalytical procedures revealed the presence of excess calcium. Its use with more heavily calcified tissues has shown that the technic gives a satisfactory relative estimate of the amount of calcium present, correlating well with quantitative estimates.

Reagents. 1. Fixative—95% ethyl alcohol. 2. 1% alizarin red S† (sodium alizarin sulfonate) in 0.10% NH_4OH, final pH 6.3-6.5. Stir 0.5 g of the dye into 45 ml of distilled H_2O so that only a few small grains of dye remain undissolved. Add 5 ml of a 1:100 dilution of C.P. 28% NH_4OH slowly with constant stirring. The pH which results is

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† Obtained from National Aniline Division, New York City.

TABLE I. Calcium Preservation by Different Fixatives.

Fixative	pH	Ca stain
10% formalin, aqueous	3.3	0 to +
saturated with marble chips	7.2	+
" " $Pb(OH)_2$	7.20	+
" " $MgCO_3$	8.9	2+
" " Na_2OO_3	10.83	2+
in .05 M veronal buffer	8.7	+ to 2+
in .2 M borate buffer	8.35	+ to 2+
95% alcohol		3+ to 4+
95% alcohol saturated with Na_2CO_3		3+ to 4+

These results were obtained in 3 series of rats inj. with 2 or 10 mg/kg of $UO_2(NO_3)_2 \cdot 6H_2$ and killed after 12-48 hr at which times the accumulation of calcium is limited, and differences therefore are apparent more easily. Samples from the same kidney were placed in each of the fixatives and handled in the usual fashion thereafter. The relative ratings represent avg of the 3 series.

6.36-6.40 as measured by a Beckman glass electrode pH meter. This solution is stable for at least one month if kept covered when not in use. After several hundred slides have been stained, the need to renew the solution will be shown by the formation of a precipitate, which is caused by the introduction of alcohol into the solution. 3. Xylol. 4. Absolute and 95% ethyl alcohols. 5. Distilled H_2O—pH *ca* 5.8.

Procedure. Specimens not exceeding 2-3 mm in thickness are fixed and dehydrated by exposure to 4 changes of 95% ethyl alcohol over a 36-hour period, followed by overnight immersion in absolute ethyl alcohol. Embedding is done directly into paraffin from which sections are cut at the desired thickness and placed without adhesive on unscratched, clean slides. Elimination of adhesive from the usual technic of mounting sections was found to be important because both gelatin and egg albumen caused fixation of the stain in a variable manner. The staining procedure is carried out at room temperature. After removal of paraffin with 2 rinses of xylol, a section is washed in 2 changes each of absolute ethyl alcohol and 95% ethyl alcohol, drained for a few seconds on a paper towel and placed in the stain for 2 minutes. Surplus stain is removed by irrigation with a gentle stream of distilled water (pH 5.8), and subsequent washing of the section in a jar of distilled water for 5-10 seconds, by which time maximum decolorization should be obtained. The stained section is dehydrated again by rinsing in 95% alcohol then absolute alcohol (2 changes of each), clarified by immersion in xylol (2 changes), and mounted in cedar wood oil. With this technic any deposits of calcium salts are revealed by an intense reddish-orange coloration, set against an almost imperceptible pink background. The specific stain should be so sharply defined that granules as small as a few micra in diameter can be seen as loci of calcification. Slight diffusion of the color occurs during immersion in the stain and subsequent wash with distilled water. This diffusion can be minimized by reduction of the staining period to one minute and of the wash to the least interval required to make the background a faint pink. Further increase of definition may be obtained by rinse with 10^{-3} M HCl in 95% ethyl alcohol (concentrated HCl 1 part, 95% alcohol 10,000 parts) for 15 seconds after the wash with water; although not generally required, this additional step may be of advantage in localizing minute foci of calcification.

Observations. Fixation of tissue. The results of fixation with a variety of solutions are shown in Table I. It had been appreciated early that acid fixatives would lead to loss of calcium salts, but not that there would be a significant difference between the neutral or alkaline formalins, as compared with 95% ethyl alcohol. As the table indicates, 95% alcohol was far superior to the other fixatives listed, and because the alkaline alcohol caused cellular swelling without enhancing the stain, neutral 95% alcohol was chosen as the fixative of choice. The considerable increase in

TABLE II. Solubility of Various Alizarate Complexes.

Salt	pH at which ppt. first appeared	pH range of max ppt.	pH range at which ppt. present	Color of alizarate
$CaCl_2$	3.90	4.28–6.75	3.90–8.05	Reddish-orange
$MgNH_4PO_4$	0	—	0	—
$MgCl_2$	0	—	0	—
$MgSO_4$	0	—	0	—
$FeCl_2 \cdot 4H_2O$	5.62	5.62–8.05	5.62–8.05	Deep purple
$Fe(NO_3)_3 \cdot 9H_2O$	5.62	5.73–6.26	5.62–8.05	Purple-black
$Ba(C_2H_3O_2)_2$	4.08	4.08–4.50	4.08–8.05	Reddish-orange to magenta
$SrCO_3$	4.50	4.50–7.38	4.50–8.05	Reddish-orange
$BeSO_4$	5.73	6.26–8.05	5.73–8.05	Magenta
$CdCl_2$	5.11	5.11–5.73	5.11–8.05	Reddish-orange to magenta
$La(C_2H_3O_2)_2$	4.50	5.73–8.05	4.50–8.05	Cherry to light purple
$Pb(C_2H_3O_2)_2$	3.50	4.08–4.50	3.50–8.05	Reddish-orange to purple
$HgCl_2$	0	—	0	—
$UO_2(NO_3)_2 \cdot 6H_2O$	4.08	4.50–5.62	4.08–8.05	Purple
$ZnCl_2$	0	—	0	—

To 2 ml of buffer (.2 M acetate buffer pH 3.50–5.87, or .1 M histidine buffer pH 5.73–8.05) in a small test tube, .5 ml of a 1% aqueous solution of the salt was added and shaken, followed by 1 uniform drop of the ammonium-alizarin staining solution, then mixed thoroughly. The tube was then centrifuged for 2 min, and precipitate and supernatant compared with a control tube containing the same buffer and alizarin, but .5 ml of H_2O in place of salt solution.

the number of stained foci with this fixative suggested the possibility that the enhanced binding of the dye might be due to a change in the tissue proteins, rather than to an improved preservation of calcium salts. This problem was first studied by noting the effect of a 1 N HCl wash, applied to kidney sections from a series of uranium-poisoned rats(7), specimens from which were preserved in both alkaline formalin and alcohol. In all instances the stained foci were shown to be present in sections which had not been exposed to the acid, whereas they were absent from the alternate sections that had received the acid wash. Further investigation of this problem was made by exposing sections to the chelating agent di-sodium-ethylene-diamine tetra-acetate which forms soluble non-ionic complexes with most di- and tri-valent metallic cations in such a manner that they no longer behave as metallic ions(9). If there were no focal areas of stain after subjecting sections to the effects of this agent, it would suggest that the ion originally responsible for combining with the stain had formed an undissociated complex with the chelating agent. Control serial sections were exposed to 0.17 M ethanolamine buffer(10) and test sections to equal parts of buffer and chelating agent (0.45 g/100 ml H_2O), brought to pH 10.4 with HCl, for 30 minutes with gentle continuous agitation. After being stained, the control slides were completely comparable with untreated stained sections whereas the test sections were either absolutely negative or at most contained a rare stained area. Finally, the observation that tissues from control (normal) rats had the same staining characteristics whether fixed in the formalins or 95% alcohol demonstrated that fixation with alcohol did not cause a general increase in the non-specific fixation of dye.

Specificity. Since spot tests with a number of magnesium salts had shown magnesium alizarate to have colors similar to calcium, it was of significance to find that calcium alizarate was almost completely insoluble in buffers over the pH range 4.0-6.8, whereas the magnesium alizarate was freely soluble (Table II). As a result the washing with distilled water, which contains traces of CO_2, eliminates interference due to the presence of magnesium. This specificity is fortunate because magnesium may accumulate in significant amounts in tissues undergoing pathological calcification(11).

Strontium, barium, beryllium, lead, and cadmium are not distinguishable from calcium (Table II); in the special problems that require consideration of their presence in the tissues, supplementary differential procedures must be employed. Iron, although it yields a purple-black precipitate when present as an ion, produces no color when present in hemosiderin, a non-ionic compound. This was verified by the absence of stain in sections of a liver‡ from a patient with hemochromatosis, despite the fact that massive deposits of hemosiderin could be demonstrated by the Prussian blue reaction.

The results with the present method have been compared with those obtained using the Von Kossa silver nitrate stain. Although long used to identify calcium, the Von Kossa procedure is not specific for that ion, but instead indicates a number of anions of which that most commonly in association with calcium, namely phosphate, is one(2). In early calcification in which analytical data(7) clearly indicated that at least part of the calcium was associated with an anion other than phosphate, the present alizarin technic revealed calcium deposits in the absence of a stain with the Von Kossa technic. In contrast, areas found positive to the Von Kossa stain were invariably positive to alizarin. Thus in each case in which an accumulation of calcium has been indicated by an independent method, it has been demonstrable with the alizarin stain.

Sensitivity of the method. When employed as a spot test on filter paper, the present method is positive for calcium above a limiting surface density of 0.02-0.002 $\mu g/mm^2$. A study with the usual 1% aqueous alizarin stain(6) revealed the present technic to be some 50 times more sensitive. This high sensitivity is dependent on proper adjustment of acidity: without neutralization a 1% solution of the dye has a pH of about 3.6 and a light brown color whereas with adjustment of the pH to 6.3 the medium is brought into the range of insolubility of the calcium alizarate and of maximum color intensity of the indicator dye. For neutralization of the stain NH_4OH was found to be superior to KOH and NaOH, since the non-specific background stain in tissue is much less when NH_4OH is employed. In use as a histochemcial procedure the stain has a greater sensitivity than manifest in spot tests. Although magnification of a field reduces the depth of color, the focal distribution of calcium deposits allows the recognition of minute areas of intense stain. From studies in which early pathological calcification of the kidneys was quantitated by direct analysis(7), it was found that the histochemical test became positive at a time when no more than 0.05 mg Ca/g tissue had accumulated; calculated for a section of 7 μ thickness this is equivalent to a surface density of 0.0004 $\mu g/mm^2$. Mordants, which have been recommended for use with alizarin dyes(5), were found to give no increase of sensitivity under the conditions of the present test. The following salts in concentrations of 0.01-10.0% were without effect: $AlCl_3$, $AlK(SO_4)_2$, $Al(OH)_3$, KCl, $K_2Cr_2O_7$, KI, $KHCO_3$, Na_2CO_3.

Among a variety of attempts to remove the pink background stain while preserving the calcium alizarate, none proved as successful as a distilled H_2O wash. Included as soaks or rinses in different concentrations before or after staining were the following: $(NH_4)_2SO_4$, urea, KOH, NH_4OH, the chlorides of Na, Hg, Mg, Zn, and Al, $La(C_2H_3O_2)_2$, 10 and 20% formalin in 95% alcohol. The relative permanence of the present stain is indicated by the oldest slides stained with the present technic: after 5 months there has been no loss in intensity of the color, and diffusion of the dye has been, at most, very slight.

Results with the stain. This stain has been used to localize pathological accumulations of calcium in the kidneys of rats poisoned with uranium, and physiological deposits in cartilage from rat tibiae;§ it has shown excellent definition of calcified areas in human kidney, aortic valve with attached myocardium, and abdominal aorta,‖ all of which showed gross calcification (Fig.1-6). In the case of the

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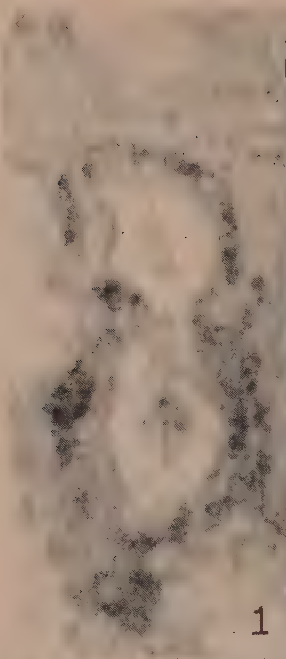
‡ Obtained from Dr. William Eisenmenger of the Hospital of The Rockefeller Institute for Medical Research.

§ Obtained from Dr. D. D. Dziewiatkowski of the Hospital of The Rockefeller Institute for Medical Research.

‖ Obtained through the courtesy of Dr. Leon Sokaloff of the Dept. of Pathology, New York University College of Medicine.



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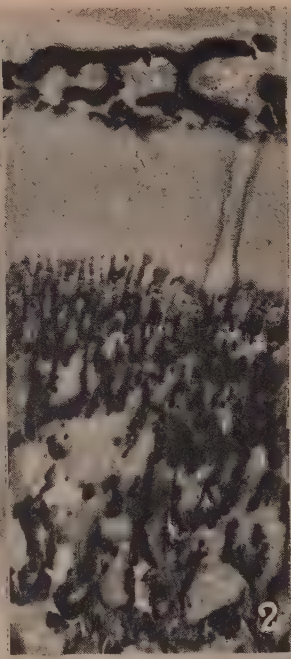

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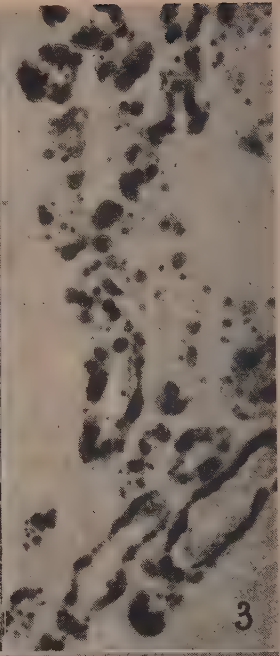

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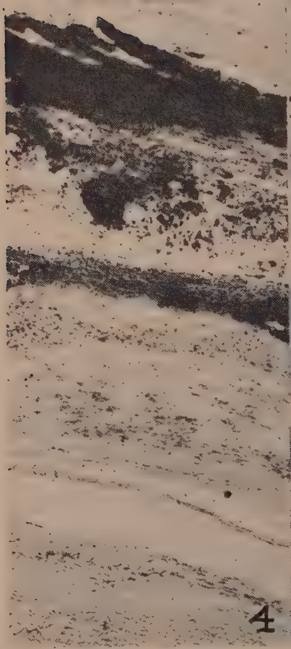

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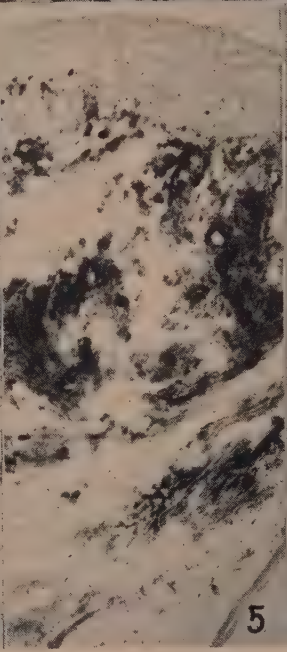

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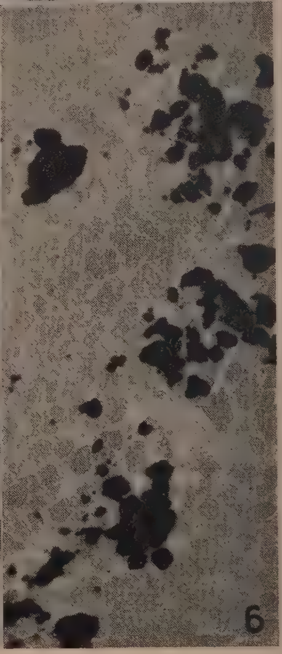

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FIG. 1. Rat kidney, showing early calcium deposits in a single proximal convoluted tubule. Alizarin. × 710.

FIG. 2. Normal 51-day rat tibia. Alizarin. × 56.

FIG. 3. Calcification in human kidney. Bellevue Hospital Autopsy #39383. Alizarin. × 128.

FIG. 4. Atherosclerotic plaque—human abdominal aorta. Bellevue Hospital Autopsy #39551. Alizarin. × 56.

FIG. 5. Calcification of aortic valve. Same case as in Fig. 4. Alizarin. × 128.

FIG. 6. Calcification of myocardial fibres. Same case as in Fig. 4. Alizarin. × 128.

uranium damaged kidneys, the method revealed minute foci of accumulation from 12-48 hours before an increase could be unequivocally demonstrated by quantitative microanalysis. The correlation of the histochemical test with quantitative analysis of the tissue was investigated in a series of sections that had been taken from the kidneys of rats after injury with uranium. On the basis of the alizarin stain 8 kidneys were ranked from greatest to least calcification. The sequence thus obtained correlated closely with the sequence based upon quantitative analysis (Table III).

TABLE III. Comparison of Tissues According to Amounts of Calcium Present.

Quantitative analysis mg Ca/g tissue	Rank	Alizarin stain Rank
8.82	1	1
6.48	2	3
5.06	3	2
2.87	4	4
2.60	5	6
1.78	6	5
1.27	7	8
1.17	8	7

The kidneys from 8 uranium-damaged rats(7) were subjected to microanalysis for calcium. Amounts of calcium found are shown at left. Sections of kidneys from each of these animals were stained with alizarin red S, and the amounts of calcium shown by this stain were ranked from greatest to least. The column on the right indicates this ranking, which is to be compared with the absolute rank, shown in the middle column.

Summary. A technic is described for histochemical definition of calcium. It is specific for calcium in the presence of magnesium, approximately 50 times as sensitive as previous methods based upon the use of alizarin, and capable of localization to within a few micra.

It is a pleasure to acknowledge the aid of Dr. Vincent P. Dole throughout the course of this work.

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Linkage Studies of Genes for Sickling and MNS Blood Groups: Negative Findings. (19662)

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(Introduced by Philip Levine.)
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Snyder, Russell and Graham(1) presented evidence of linkage between the genes for MN blood types and the gene for sickling of erythrocytes. Subsequent studies by Snyder, Clark and Moore(2), and Snyder(3) gave additional supporting evidence for linkage between these two genes. The above studies were based on a total of 85 families, 12 of which were usable for linkage studies. Race *et al.*(4), in studies on the MN and S groups in 123 English families observed close linkage between the genes for these two blood groups, no recombination being observed in 82 children capable of disclosing it. The prediction of the allele s by Sanger and Race (5) and the subsequent discovery of an anti-s serum by Levine *et al.*(6), further supported the concept of linkage between S and MN. While the present investigation was under way, Neel and Hanig(7) in their study on the inheritance of the MN and S factors in American Negroes, found no evidence of recombination among 18 children of 5 matings, thus lending confirmation to the observations of Race *et al.*, in a different population group.

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‡ Deceased July 14, 1952.

The present investigation was undertaken with the hope of confirming the above studies and determining the relative position of the genes for MN, S and sickling.

Procedure. This study is based on an analysis of 325 individuals from 55 Negro families of which 52 are complete, each consisting of father, mother, and one or more children. In addition to the determinations for MN, S and sickling, tests for the Rh (D,C,E) factors and the ABO blood groups were also performed in order to exclude as many illegitimate children as possible. Only about one-third of the families lent themselves to the study of linkage between MN, S and sickling. The data were subjected to analysis by Finney's method for the detection of linkage(8). Several of Finney's mating types were used and all gave comparable results. Only the following mating types will be reported here:

Finney Type				
20	MM or NN	si si[*]	×	MN Si si
21	MN	si si	×	MN Si si
22	MN	Si si	×	MN Si si

For the 17 families analyzed for linkage between MN and sickling the total score, $\Sigma(\lambda)$, was found to be –2. The variance of the score, $\Sigma(\kappa)$ was found to be 51. Because $\Sigma(\lambda) = -2$, is less than $1.64\sqrt{\Sigma(\kappa)} = 11.71$, no linkage could be demonstrated.

Although the quantity of information available for analysis of linkage between S and sickling, and MN and S is small, it will be included here for the sake of completeness. For the 5 families analyzed for linkage between S and sickling, the $\Sigma(\lambda)$ amounted to $+3$ and the $\Sigma(\kappa)$ to 13. The $\Sigma(\lambda)$ was calculated to be less than $1.64\sqrt{\Sigma(\kappa)}$ and consequently again no linkage could be shown. For the 5 families analyzed for linkage between MN and S, $\Sigma(\lambda)$ was $+9$ and $\Sigma(\kappa)$ 19. Therefore, because $1.64\sqrt{\Sigma(\kappa)}$ is exceeded by $\Sigma(\lambda)$, linkage is indicated.

The entire family material was submitted to Drs. R. R. Race and Ruth Sanger[†] who very kindly analyzed the data according to the u-statistics of Fisher(9). The families were scored by them in two ways: 1) assuming that sickle cell anemia is the homozygous condition and the sickle cell trait the heterozygous (10). 2) not making the above assumption and judging heterozygotes as those persons with one or more negative children. When the mating was sickler x sickler, only the negative children were scored. Thus 23 families were analyzed for linkage between the MNS system as a whole and sickling, using evidence from both homozygotes and heterozygotes(1). This study yielded values of $\Sigma(\lambda) = +9$ and $\Sigma(\kappa) = 117$. As the $\Sigma(\lambda) = +9$ does not exceed $1.64\sqrt{117} = 17.74$, linkage could not be demonstrated. Without making a distinction between the heterozygote and homozygote(2), 17 families were recalculated for linkage with MNS and values of $\Sigma(\lambda) = +4$, and $\Sigma(\kappa) = 106$ were found. Because $\Sigma(\lambda) = +4$ is less than $1.64\sqrt{106} = 16.89$, no linkage was indicated.

All pedigrees, a frequency study of MNS and sickling on unrelated Negroes, as well as methods for the detection of sickling and details of crossover analysis will be reported elsewhere.

Summary. Analysis of data supplied by 55 Negro families comprising 325 kindred, failed to reveal linkage between the MNS blood group genes and the gene for sickling. These findings are not in accord with those of Snyder and coworkers. Thus an example of autosomal linkage, for which Snyder *et al.* presented evidence, is now in doubt and warrants further investigation. Data ascertainable for linkage calculations between the blood group S and sickling, and MN and S were very small.

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[*] non-sickler

[†] Lister Institute, London.

Linkage between S and sickling could not be found. The blood groups MN and S afforded evidence for linkage thus fitting into the very considerable evidence on this point for Caucasians.

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Effect of Dihydroergotamine on Epinephrine Response in the Hind-Limb of Dogs.* (19663)

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The vasoconstrictor action of exogenous epinephrine can be blocked readily with the various ergot alkaloids(1). Conclusions based primarily on changes in the systemic blood pressure indicate that these alkaloids will not block the vasodilator response of epinephrine. On the other hand the ergot alkaloids will block the inhibitory action of epinephrine on isolated intestinal strips(2-4). There may be no similarity between the mechanism of inhibition of the intestine by epinephrine and the vasodilator response on the vascular system. Nickerson(1) objects to the use of the intestine to investigate the effect of ergot compounds on the inhibitory action of epinephrine because of the presence of intramuscular parasympathetic ganglia in this tissue and the potentiation of the cholinergic response with some of the ergot alkaloids. The experiments reported here were designed to determine the influence of ergot alkaloids on the response of the vascular system of the hind-limb of dogs to intra-arterially injected epinephrine.

Method and material. Dogs of either sex and weighing between 12 and 23 kg were anesthetized with 25 mg/ kg of thiopental and 40 mg/kg of chloralose. Through a mid-line abdominal incision, all branches of the lower 5 to 8 cm of the abdominal aorta and inferior vena cava, except the common iliac artery and vein for the experimental leg, were ligated. Blood flow from the experimental hind-leg was measured with a bubble flowmeter inserted into the common iliac vein. The blood was returned to the inferior vena cava by way of the opposite common iliac vein. The temperature in the flowmeter was maintained at 37°C by means of a water jacket. Blood pressure was recorded from the common iliac artery of the opposite leg and the heart rate was recorded with the electrocardiograph. The intra-arterial injections were made through a cannula inserted into the middle sacral artery. Intravenous heparin was given periodically throughout the experiment. Fresh solutions of synthetic l-epinephrine,† dihydroergotamine methanesulfonate and Hydregine (CCK-179) were prepared in a 0.9% sodium chloride solution. All intra-arterial injections were of a 2 ml volume and were given over a one minute period.

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* This study was aided by a grant from funds obtained through Initiative 171, State of Washington.

† Synthetic l-epinephrine kindly supplied by Stansbury Chemical Co., Seattle; the dihydroergotamine and Hydregine supplied by Mr. Harry Althouse, Sandoz Chemical Co.

TABLE I. Summary of Changes in Blood Flow Response of Hind-Limb of Dogs to Epinephrine Injected Intra-arterially at Various Time Intervals During a 3-Hr Experimental Period. First injection considered the control response.

Epinephrine, γ/kg I.A.	Constriction	Dilation	
	No reduction	Partial reduction	No reduction
1	+	+	
	+	+	
	+	+	
	+		+
3	+	+	
	+	+	

Partial reduction = 25% to 75% decrease in epinephrine response from control.

No reduction = 25% or less decrease in epinephrine response from control.

Results. The procedure and calculation of the results for the experiments were as follows: Approximately 30 to 45 minutes after the bubble flowmeter was incorporated into the system, control blood flow responses were obtained to the intra-arterial injections of one and/or 3 γ per kilo of epinephrine. These quantities of epinephrine produced little or no change in the systemic blood pressure and heart rate. The epinephrine injections were repeated at least twice between ½ to 2½ hours after the intra-arterial injection of 20 γ per kilo of dihydroergotamine. For each epinephrine injection there was calculated the maximal per cent changes in blood flow from the control pre-injection rate. In order to compare the experimental data, this method of calculating the results was used because the pre-injection flow rates would vary throughout the experimental period. The maximal per cent changes in blood flow with each epinephrine injection following the dihydroergotamine were calculated in the same manner and then compared with the control epinephrine responses.

Table I shows that in the absence of dihydroergotamine repeated intra-arterial injections of epinephrine result in no reduction in the vasoconstrictor response and a partial reduction in the vasodilator response. Table II shows that dihydroergotamine completely blocked the vasoconstrictor action of epinephrine but failed to completely block the vasodilator action of epinephrine in the majority of experiments. In 2 additional experiments, Hydregine (CCK-179) in a dose equal to that used for dihydroergotamine resulted in complete blockade of epinephrine vasoconstrictor action but only partial blockade of the vasodilator action.

In the experiments with dihydroergotamine, this substance produced a transient increase in blood flow during the injection, otherwise it had no effect on flow rates.

Discussion. Some of the experiments showed that dihydroergotamine produced a partial decrease in the vasodilating response to epinephrine. This effect of dihydroergotamine could be due to factors other than its direct action on the vasodilator epinephrine mechanism. A decrease in the vasodilator action of epinephrine could be due indirectly to the blocking of the vasoconstrictor effect of epinephrine thereby modifying the concentration and the duration that the epinephrine would remain in contact with the receptor mechanism in the leg vessels. This could explain the fact that the larger dose of epinephrine resulted in a definite systemic effect on heart rate and blood pressure after dihydroergotamine and little or no systemic effect before dihydroergotamine.

Another factor responsible for the decreased vasodilator response of epinephrine could be due to a progressive decrease in the response of the vascular system to repeated injections of epinephrine during the experiment. The present experiments showed no change in the normal vasoconstrictor response of intra-arterial injected epinephrine and a partial reduction of the vasodilator response. The partial blockade of the epinephrine vasodilator response following dihydroergotamine in some of the experiments could be due to the progressive decrease of the normal vasodilator response of repeated injections of epinephrine.

In some of the experiments with dihydroergotamine, there was an increased vasodilator action of epinephrine which subsequently returned toward normal at the end of the experiment. The initial increase in the vasodilator action could be due to the blocking of the vasoconstrictor mechanism thereby unmasking the vasodilator response. The subsequent decrease in the vasodilator response could represent a definite effect of dihydro-

TABLE II. Summary of Effect of Dihydroergotamine on Blood Flow Response to Epinephrine Injected Intra-arterially in Hind-Limb of Dogs. Epinephrine injected between ½ and 2½ hr after intra-arterial injection of 20 γ/kg of dihydroergotamine.

Epinephrine, γ/kg I.A.	Constriction Complete block	Dilation Complete block	Dilation Partial block	Dilation No change	Dilation Incr. response
1	+		+		
	+				+
	+	+			
	+		+		
	+	+			
	+				+
	+			+	
3	+				+
	+		+		
	+				+
	+		+		
	+				+

Complete block = >75%
Partial " = 25% to 75%
No change = 25% or less
} decrease in epinephrine response after dihydroergotamine.

ergotamine on the epinephrine dilator mechanism or a recovery of the vasoconstrictor action.

Conclusions. In blood flow experiments in the hind-limb of dogs, dihydroergotamine completely blocks the vasoconstrictor action of epinephrine but fails to uniformly block the vasodilator action of epinephrine.

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Effect of Dietary Molybdenum Upon Chicks and Poults.* (19664)

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When cattle and sheep graze on pastures in which the molybdenum exceeds approximately 10 p.p.m., toxic symptoms may appear which include diarrhea, anemia, weight loss and a change in the color of the hair or wool(1,2). Rats and rabbits can tolerate molybdenum better than ruminants although 500 p.p.m. caused a definite growth retardation and 5000 p.p.m. resulted in the death of all of the rats in one experiment(3-6). Copper is capable of counteracting the symptoms of molybdenum toxicity in both ruminants and rats. The present studies were undertaken to determine the effects of feeding various levels of molybdenum and copper to chicks and poults.

Experimental. Exp. 1, 2 and 3 were conducted with S. C. White Leghorn male chicks while Bronze poults were used in Exp. 4, 5 and 6. Practical chick and poult starting rations were used as basal rations to which various levels of sodium molybdate dihydrate and copper sulfate were added. The basal rations contained 1.5, 1.5, 1.3, 4.0, 6.0, and 1.5 p.p.m. molybdenum in the successive trials. The copper in the basal ration was determined only in Exp. 2, 3, 5 and 6 in which it was found to be 9.9, 8.2, 20.0 and 10.0 p.p.m., respectively. The birds were housed in electrically heated batteries with wire floors and were supplied feed and water *ad libitum*. The birds were weighed at frequent intervals. There were 7, 13, 10, 6, 7 and 10 one-week-

TABLE I. Effect of Molybdenum and Copper in the Ration upon Growth of Chicks and Poults. Avg gain (g).

Chicks			Poults		
Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6
Days					
23	28	32	34	30	31
229 (0, 0)*	281	76	594	620	484
	260 (50, 0)				
244 (85, 0)					
	225 (100, 0)	323		617	516
217 (170, 0)			460		
	237 (200, 0)	289		612	374
	251 (200, 100)				481
194 (255, 0)			397		
199 (255, 150)			418		
	204 (300, 0)	279		481	
		304 (300, 100)			
		305 (300, 200)			
		306 (300, 300)			
174 (340, 0)			293		
193 (340, 200)			398		
	199 (400, 0)				
	236 (400, 200)				
	203 (400, 400)				

* p.p.m. added molybdenum and copper respectively.

TABLE II. Effect of Molybdenum and Copper in the Diet upon Hemoglobin, Molybdenum and Copper in the Blood of Chicks and Poults.

Chicks		Poults							
Hemoglobin, % Exp. 2	Mo. in blood, p.p.m. Exp. 2	Hemoglobin, % Exp. 5	Hemoglobin, % Exp. 6	Cell vol, % Exp. 6	Mo. in blood, p.p.m. Exp. 4	Mo. in blood, p.p.m. Exp. 5	Mo. in blood, p.p.m. Exp. 6	Cu. in blood, p.p.m. Exp. 5	Cu. in blood, p.p.m. Exp. 6
8.9 (0, 0)*	1.2	13	10.4	37.5	2.1	.1	.4	1.9	1
8.5 (50, 0)	12.9								
8 (100, 0)	26.4	12	10.6	36.2		3.2	13	1.5	1
					4.3 (170, 0)				
8.4 (200, 0)	34.1	12.1	10.9	35.6		8.2	12.8	1.8	1.8
8.3 (200, 100)	24.3		11.3	37.6			12.9		1.2
					18 (255, 0)				
					9.7 (255, 170)				
8.8 (300, 0)	32.2	12.4				15.5		1.8	
					24.7 (300, 0)				
					7.2 (300, 200)				
9.3 (400, 0)	16.7								
9.2 (400, 200)	28.6								
9.9 (400, 400)	21.6								

* p.p.m. added molybdenum and copper respectively.

old birds started per group in the successive trials. Hemoglobin determinations were made on some of the birds at the end of some experiments by the method of Matterson(7). Molybdenum was determined by a slight modification of the method of Marmony(8) and copper by the method of Association of Official Agricultural Chemists(9). Samples of excreta were saved for moisture determination by leaving a waxed paper overnight on the dropping pan in the battery and obtaining a sample from the accumulated excreta the next morning.

Results. With but one exception in Exp. 1, there was generally a reduction in growth of chicks as the molybdenum of the ration was increased (Table I). The growth reduction was approximately 25% when 300 p.p.m. molybdenum was fed. When copper was added to the rations containing molybdenum there was an improvement in growth, although it was usually only slight. With one exception the growth of poults was also decreased approximately as much as chicks when molybdenum was added to the ration. The addition of copper to the ration improved

TABLE III. Effect of Molybdenum and Copper in the Ration upon Moisture in Excreta of Chicks and Poults.

Moisture in droppings, %		
Chicks		Poults
Exp. 2	Exp. 3*	Exp. 6†
77 (0, 0)‡	62	57
75 (50, 0)		
75 (100, 0)	68	67
64 (200, 0)	61	71
68 (200, 100)		67
59 (300, 0)	67	
	56 (300, 100)	
	53 (300, 200)	
	56 (300, 300)	
54 (400, 0)		
70 (400, 200)		
68 (400, 400)		

* Avg of 2 separate collections.

† Avg ” 3 ” ”

‡ p.p.m. added molybdenum and copper respectively.

growth in the 3 groups on which it was tested.

Hemoglobin was determined in one chick and 2 poult experiments but showed no variation which could be attributed to the levels of molybdenum and copper in the ration (Table II). Blood molybdenum increased enormously as the molybdenum in the ration increased in both chicks and poults. When copper was added, a slight reduction usually was noted in the blood molybdenum levels. In Exp. 6, analyses on plasma and whole blood samples of poults indicated that approximately 90% of the molybdenum was in the plasma. The level of copper in the blood of poults did not appear to be related to the molybdenum and copper levels of the ration under our conditions (Table II).

The data for the moisture content of the excreta (Table III) show no consistent differences as the rations varied in molybdenum and copper content. The results may be interpreted to show that diarrhea is not a symptom of molybdenum toxicity in chicks and poults.

In the 3 experiments with poults there was no evidence of faulty pigmentation.

Discussion. Chicks and poults seem to resemble rats in their molybdenum tolerance (3-6). High levels are needed to depress growth and anemia has not been noted. The response in ruminants seems much different since much lower levels may be detrimental (2). The simultaneous feeding of copper improved the growth of chicks and poults as it does with rats and ruminants, but it did not overcome the effect of molybdenum completely.

No diarrhea was noted in poults and chicks fed high-molybdenum rations. This is in contrast to ruminants on a high molybdenum pasture; however, cattle and sheep have been fed dry hay rations containing 200 p.p.m. with no diarrhea(12).

Cattle and sheep fail to form hair pigment properly when they are fed molybdenum and dark colored animals become light colored. Intravenous injections of molybdenum, however, did not cause a change in pigment formation in sheep while the same quantity fed produced a color change in a few days(12). In poults, lysine has been shown to be necessary for normal feather pigment formation (11) and approximately 2 weeks are required for the effect of feeding a lysine-low ration to be shown in the feathers(10). Molybdenum had no effect on pigmentation in turkey poults for a period of over 4 weeks. This suggests that either a different mechanism is involved in pigment formation in turkeys than in ruminants or the level of copper was adequate in the poult rations while it may have been marginal in the experiments with ruminants.

Comar *et al.*(4) have concluded that the unavailability of other elements due to molybdenum complex formation probably cannot account alone for the toxic action of molybdenum. This view would seem to apply to chicks and poults. Although no attempt was made to reduce the copper content of the rations used, there was no indication of a copper deficiency in which one might expect to observe anemia in the birds. The results suggest that the growth depressing effect of molybdenum is due to the presence of the element itself in the tissues. The slightly beneficial effect of copper may have been due to a reduction of the tissue molybdenum level, although the blood molybdenum was not reduced in all instances. Barshad(13) has shown that the uptake of molydenum by plants is much greater in forage material than in the seeds. Since poultry rations consist mainly of seeds, and chicks and poults have a high tolerance for molybdenum, there is little likelihood that molybdenum poisoning will ever be a practical problem with poultry.

Summary. Growth of chicks and poults was depressed by approximately 25% by the addition of 300 p.p.m. molybdenum to their rations. The addition of copper to the rations containing molybdenum caused slightly improved growth. There was no evidence of anemia or diarrhea in birds on the high molybdenum diets. Poults fed high molybdenum rations for over 4 weeks had normal feather pigmentation.

We are grateful to Dr. Harold Goss for advice and assistance in the molybdenum and copper analyses and to Mr. R. L. Smith for preliminary investigations of this problem.

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A Method for Quantitative Identification of Canine Erythrocytes. (19665)

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Studies of erythrocyte metabolism and function in the dog have been hindered by the lack of a reliable quantitative method of red cell identification. Hamilton(1) and Young *et al.*(2) have recently alluded to serologic, and Harrison *et al.*(3) to isotopic technics. In the present communication, we report a method for the quantitative identification of canine erythrocytes by differential agglutination utilizing lyophilized anti-sera. Young and his collaborators(2,4) have described 6 erythrocyte agglutinogens in dogs. These have been arbitrarily designated as A, B, C, D, E, and F. These canine agglutinogens do not correspond to human agglutinogens of similar terminology. In random examination of a large group of dogs, the A agglutinogen was found in about 63% of dogs, the B in about 5%, the C in 99%, the D in about 30% and the E in 80%. The incidence of the F agglutinogen has not been reported. Presumably there are other agglutinogens not yet identified. Christian *et al.*(4) have observed that variants of the A agglutinogen exist. These have been designated A′ and are present in about 40% of A dogs. The A-A′ differences are partly qualitative and are thought to resemble human D-D^u differences more closely than human A_1-A_2 differences.

Isohemagglutinins have been demonstrated in dog sera. Anti-D has been observed as a naturally occurring antibody, while anti-A,-B,-C,-E, and -F have been demonstrated only in dogs transfused with erythrocytes containing the respective factors.

Methods. The A agglutinogen is strongly antigenic, and high titre anti-A sera can be produced by multiple transfusions of A cells into dogs whose erythrocytes do not contain the A agglutinogen. However, at the same time there is produced an hemolysin which is not specific for A cells. It was found that

TABLE I. Survival of Transfused Erythrocytes in a Normal Dog.

Day of study	Blood vol, ml	Plasma vol, ml	Circulating red cell mass, ml	Recipient cells, ml	Infused cells, ml
			Before infusion		
20	1220	705	515	515	—
16	1200	720	480	480	—
1	1285	770	515	515	—
Mean	1235	732	503	503	—
			After infusion		
0	1246	732	516	427	89
5	1270	750	520	436	84
17	1285	770	515	448	67
35	1300	750	550	500	50
38	1310	730	580	534	46
69	1256	715	540	517	23

this hemolysin could be removed by absorption with appropriate red cells. In our red cell survival studies, it was decided to use only C, D, or CD dogs as donors and as recipients, AC, AD, and ACD dogs (with or without E and F agglutinogens). Thus, no known iso-antibodies would be developed to the transfused cells. To remove the hemolysin, fresh CD red cells which have been washed 3 times in isotonic sodium chloride solution were added to anti-A serum (titre 1:128 or greater) in which complement had been inactivated by incubation for one-half hour at 56°C. The mixture was kept at room temperature (about 25°C) for one-half hour. The serum was then removed after centrifugation. The addition of washed CD cells (saline, in amount about 10 times the volumes of the red cells, was used for each washing) was repeated until there was no hemolysis of CD cells. Usually 3 additions of CD cells were sufficient to absorb the hemolysin. Hemolysis was detected as follows: An autologous serum suspension of CD cells was made; one side of a counting chamber was filled with this mixture. At the same time 0.1 ml of the suspension was added to 0.1 ml CD absorbed anti-A serum in a 10 x 75 mm tube. This second mixture was allowed to stand at a temperature of about 25°C for 15 minutes and was then centrifuged for 3 minutes at 1000 RPM. The tube was shaken vigorously against the palm of the hand to free the cells, and the other side of a counting chamber was flooded with a sample of the supernatant mixture. The entire red cell field was counted on both sides of the chamber. In the absence of hemolysis, the count in the first chamber was twice that in the second chamber. After anti-A sera had been adequately absorbed with CD cells, they were shell-frozen at −70°C and lyophilized. The dried sera were pulverized and mixed into an homogenous mixture and stored in a desiccator over calcium chloride at 4°C. To reduce the quantity of blood required for serial observations of survival of transfused red cells, it was decided not to use autologous sera for making red cell suspensions for differential agglutination measurements. Isotonic saline as a suspending medium was not suitable since agglutination under these conditions was incomplete and free counts of 20 to 25% were obtained. Bovine albumin (10 to 20% in saline) resulted in excessive rouleaux formation and incomplete agglutination (free counts of 8 to 12%). Homologous serum from dogs with the same red cell types as the recipient dogs proved satisfactory as a suspending fluid. Free counts were negligible, and *in vitro* mixtures of red cells could be counted within an error of about 5%.

The differential agglutination counting technic is as follows: Oxalated blood from a recipient dog (or an *in vitro* mixture) is taken in a red cell pipette to the 0.5 mark and suspended in one ml fresh serum from an ACD dog. A total count is done on this suspension and, from the same pipette, 3 to 4 drops of the cell suspension is added to a 10 x 75 mm test tube in which dried absorbed anti-A serum has been placed. Enough of the

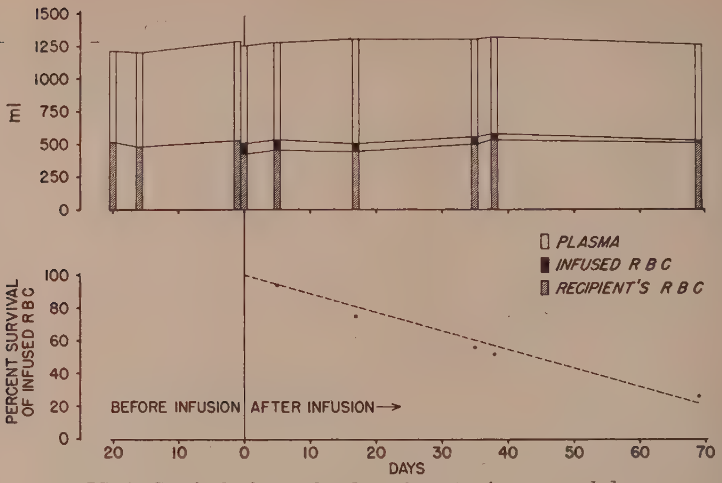

FIG. 1. Survival of transfused erythrocytes in a normal dog.

dried serum is used to cover the bottom of the test tube to a height of about 1/16 of an inch. No significant change in volume of the added cell suspension occurs. The tube is allowed to stand for 15 minutes at a temperature of about 25°C. It is then centrifuged for 3 minutes at 1000 RPM. The tube is shaken against the palm of the hand to free the cells and sufficient chambers are filled to enable the counting of 1000 unagglutinated cells. The counting is done immediately; a delay of over thirty minutes leads to non-specific hemolysis.

Experimental. The survival of infused normal canine erythrocytes was studied in four healthy mongrel dogs maintained on a constant nutritionally adequate diet. Serial measurements of nitrogen intake, urinary and fecal nitrogen output, various liver functions (serum bilirubin, bromsulfalein retention, prothrombin time, and thymol turbidity), iliac crest bone marrow morphology, and the peripheral red and white cells were made. Plasma volumes were measured with T-1824 (5) and the circulating red cell masses calculated(6). After a series of control observations, approximately 20% of the blood of the recipient dog was withdrawn and immediately replaced with an equal quantity of blood just withdrawn into acid-citrate-dextrose from the donor dog. Replacement transfusions were carried out to minimize effects of the procedure on erythropoiesis(7,8). Serial measurements of plasma volumes, and red cell masses, donor and recipient, were made. These data of a representative study are presented in Table I and Fig. 1. The destruction of the infused cells proceeded at a rate of about 1% per day. This is similar to the figures mentioned by Young *et al.*(2), Hamilton(1) and Harrison *et al.*(3). No changes in bone marrow morphology or peripheral leucocytes were noted. Nitrogen balance was not significantly changed.

Summary. A procedure for the quantitative determination of injected canine erythrocytes by a differential agglutination technic utilizing lyophilized type-specific anti-sera is described. The method was applied in a study of erythrocyte survival after transfusion of blood. Approximately 20% of the blood of the recipient dogs was withdrawn and immediately replaced with an equal volume of donor blood. Determinations were made of red cell volume, plasma volume, donor and recipient red cell counts. Serial examinations of bone marrow and peripheral leucocytes were also made. Destruction of infused cells proceeded at a rate of about one per cent per day. No changes in bone marrow morphology or peripheral leucocytes were noted. Nitrogen balance was not significantly changed.

The opinions are those of the authors and do not necessarily represent the official views of any governmental agency.

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Comparative Effectiveness of Five Newly Synthesized Arsenical Compounds in Treatment of Leukemia in Mice. (19666)

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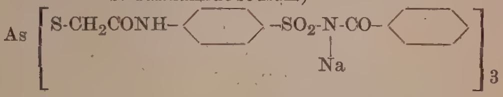
Arsenic in the form of potassium arsenite (Fowler's solution) is an effective therapeutic agent in chronic myelogenous leukemia. Theoretically the arsenic acts as a general poison by combining with the thiol compounds present in protoplasm, probably SH components of the pyruvate oxidase enzyme system(1). In chronic myelogenous leukemia there seems to be a selective action on the myeloid cells(2). It is our impression that this selective action of Fowler's solution on myeloid elements is greater than that of other agents such as urethane and irradiation so that fewer instances of concurrent depression of the other marrow elements occur. It therefore seemed desirable to study the effects of other arsenical compounds in an effort to find one of greater therapeutic efficiency. In this study 5 newly synthesized trivalent organic arsenical compounds (with the arsenic not attached to a benzene ring) were compared along with potassium arsenite for their ability to prolong the lives of mice affected with transmitted leukemia. In planning the types of organic arsenic compounds to be tested, it seemed desirable to so modify the organic "carrier radical" that transport across cell membranes might significantly differ with each type. The identifying MC numbers, structures and names of the compounds tested are shown below:

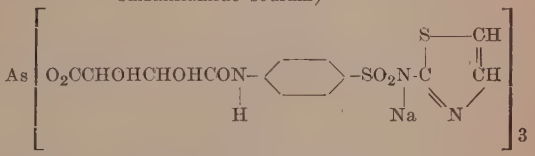
MC 2186—Arsenic-tris-(N^4-mercaptoacetyl-N^1-benzoyl sulfanilamide sodium)



MC 2767—Tris-(β-Diethylaminoethylthio)arsine hydrochloride

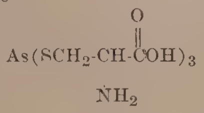
$$\left[(C_2H_5)_2\text{-}NCH_2CH_2S \right]_3 As \cdot 3\,HCl$$

MC 2768—Arsenic-tris-(N^4-tartaryl-N^1-2-thiazolylsulfanilamide sodium)



MC 2864—Tris (β-hydroxyethylthio) arsine
$(HOCH_2CH_2S)_3$ As

MC 2865 — Tris (β-carboxy-β-aminoethylthio) arsine



$$As(SCH_2\text{-}\underset{NH_2}{CH}\text{-}\overset{O}{\overset{\|}{C}}OH)_3$$

In Type 1 (MC 2186 and 2865), arsenic is bound to sulfur as part of an anion; in Type 2 (MC 2767), arsenic is bound to sulfur as part of a cation; in Type 3 (MC 2864), arsenic is bound to sulfur in a neutral molecule;

TABLE I. Results of Toxicity Studies in Mice.

Drug	Approx. molecular wt	Arsenic atoms per molecule	Max tolerated dose, mg/kg	Dosage used in therapeutic exp., mg/kg	Relative amt of arsenic per therapeutic dose; potassium arsenite = 1
Potassium arsenite	146	1	15	7.5*	1
MC-2186	1522	1	80	80	1
2767	748	1	40	40	1
2768	1633	1	320	320	4
2864	306	1	10	10	1
2865	433	1	40	20-40†	1-2

* This dosage was used so that the studies could be roughly compared with similar studies done by Burchenal *et al.*(3).

† Four animals in exp. 2 received only 20 mg/kg.

and in Type 4 (MC 2768), arsenic is bound to oxygen as part of an anion. Thus, we were afforded an opportunity to gain some insight as to the chemical structural requirements for activity in the treatment of experimental leukemia.

Materials and methods. Mice of the Ak strain were used in all experiments. The toxicity studies were performed with mice of the Ak strain obtained from the Rockland Farms. All therapeutic studies, however, were performed using Ak_m mice inbred in our own laboratory. Practically all of this strain of mice die of leukemia following the intraperitoneal injection of leukemic cells from another mouse. The mice used in the experiments varied in age from 6 to 9 weeks. Leukemia does not occur spontaneously until later in life.

Tumors. Two transmissible tumors were used. 1. Lymphoid leukemia 1527. This developed spontaneously in a mouse of the Ak_m strain. A subcutaneous tumor of lymphoid tissue could be produced by injecting the cells subcutaneously. After intraperitoneal injection the animals all died of a generalized leukemia with a marked elevation of the white blood cell count and the presence of many lymphoblasts in the peripheral blood. There was a generalized lymph node enlargement and a moderate enlargement of the spleen and sometimes the liver. The tumor was observed through several transmissions before it was used so that its natural history could be studied.

2. Myeloid leukemia 1067. This also developed spontaneously in a mouse of the Ak_m strain. The mouse was called to our attention by the relatively large size of the spleen as compared to the lymph nodes. Blood smears from the original mouse as well as from mice to which the tumor was transmitted revealed a very high white blood cell count, many of the cells being immature and containing granules (promyelocytes). This impression was

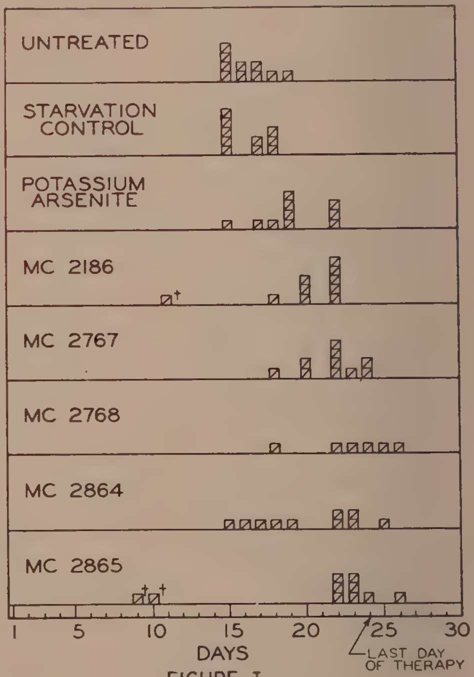


FIGURE I
EXPERIMENT I
LYMPHOID LEUKEMIA

▨ — 1 DEAD MOUSE
† — NOT INCLUDED IN CALCULATIONS

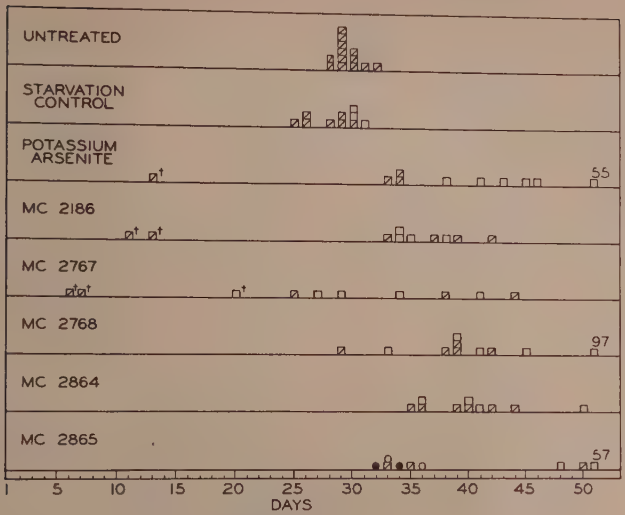


FIGURE II
EXPERIMENT 2
MYELOID LEUKEMIA

▨—●—1 DEAD MOUSE
CIRCULAR SYMBOLS MARK MICE THAT RECEIVED HALF THE USUAL DOSE OF THE DRUG
OPEN SYMBOLS MARK MICE THAT RECEIVED THERAPY BEYOND THE REGULAR 10 INJECTIONS
† — NOT INCLUDED IN CALCULATIONS

confirmed by staining the smears with peroxidase stains. The cells were not used in the experiments until they were transferred several times in order to be sure that no appreciable change occurred in the characteristics of the cells.

Compounds tested. All of the compounds were dissolved in physiological saline. In the case of MC 2865 it was necessary to add a few drops of NaOH to effect solution. The concentrations were adjusted so that the volume of injected material per mouse was in the range .04 to 0.32 ml in the toxicity experiments and 0.06 to 0.24 ml in the therapeutic experiments. Within any one group of animals the volume of injected material was almost constant.

The methods used were modifications of those described by Burchenal *et al.*(3). The maximum tolerated dose of the therapeutic agents for Ak mice was first determined. For each drug tested at least 15 mice were used, 3 mice for each of 5 dosages. Usually the doses selected were from the following: 10, 20, 40, 80, 160, 320, 640, and 960 mg/kg. In some cases intermediate doses were also tested. The mice were injected intraperitoneally daily for 7 days. The animals were weighed daily. Appreciable loss of weight and death were considered signs of toxicity. The drugs were then tested for their ability to prolong the lives of mice carrying transmitted leukemia. Eight groups consisting of 10 mice each were used in each of 2 experiments. The lymphoid leukemia tumor was used in the first experiment and the myeloid leukemia in the second. In each experiment the following groups were studied: untreated control, starvation control, Fowler's solution treated, and the 5 groups treated with the new arsenicals. The starvation control group were fed one g of bread wet with milk plus 0.75 g of chow daily per mouse. Water was given *ad lib.*(4). One million cells (0.1 cc) of a saline suspension of minced subcutaneous tumor in the first experiment and of minced spleen and lymph node in the second experiment were injected intraperitoneally into all of the mice. Forty-eight hours later the animals were divided into the 8 groups and therapy or the starvation diet was started. The drugs were injected intraperitoneally 3 times a week (MWF) for a total of 10 injections. In the second experiment a few of the animals of each group were

TABLE II.

Group	No. of mice	Survival time, days		Stand. error, Sm
		Range	Mean	
		First exp.		
Combined untreated control	20	15-19	16.3	.307
Potassium arsenite	10	15-22	19.2*	.727
MC-2186	9	18-22	20.9*	.485
2767	10	18-24	21.7*	.597
2768	6	18-26	23 *	1.157
2864	10	15-25	20 *	1.087
2865	8	22-26	23.1*†	.481
		Second exp.		
Combined untreated control	23	25-32	29	.073
Potassium arsenite	9	33-55	41 *	.845
MC-2186	8	33-42	36.5*†	.410
2767	7	25-44	34 *†	1.127
2768	10	29-97	44.2*	2.010
2864	10	35-50	40.3*	.469
2865	9	32-57	39.8*	1.094

* These means are significantly different from the mean of the combined untreated control group on the 1% level (Fisher and Yates) (5).

† These means are significantly different from the mean of the potassium arsenite treated group on the 1% level.

continued on therapy beyond the 10 injections. These are so marked on the chart. The mice were weighed frequently. In a few cases the dosage of the drug was reduced if it was felt that the animal was developing drug toxicity. At death the animals were examined grossly for evidences of disease. The statistical significance of the results was calculated by using a method described by Fisher and Yates(5) for determining the significance between 2 Student distributions. The treated groups were compared with the combined untreated control group. The groups treated with the new arsenicals were compared to the potassium arsenite treated group.

Results. Table I shows the results of the toxicity studies. It will be seen that mice can tolerate about 4 times as much arsenic when given in the form of MC 2768 as compared to any of the other compounds.

Fig. 1 and 2 demonstrate the results of the 2 therapeutic experiments. In the first experiment (lymphoid leukemia) the 3 mice that died before the 12th day were losing weight and probably died from drug toxicity. These were not included in the significant statistics. Since there was no appreciable difference between the untreated control group and the starvation control group, these together were considered the combined untreated control group. As can be seen in Table II, there is a significant difference between each of the treated groups and the combined untreated control group. When each of the groups treated with the new arsenical compounds is compared to the group treated with potassium arsenite, only the group treated with MC 2865 shows a difference which is statistically significant (probability $<.01$).

In the second experiment (myeloid leukemia) 6 animals which died before the 21st day were not included in the significance calculations since they showed signs of severe drug toxicity. The untreated control group and the starvation control group were considered as one combined untreated control group as in the first experiment. All of the treated groups are significantly different from the combined untreated control group (Table II). Two of the groups treated with the new arsenical compounds (MC 2186 and MC 2767) are of significantly less value when compared to the group treated with potassium arsenite. None of the drugs is significantly better than the potassium arsenite.

Summary. While all of the treated groups of animals showed a statistically significant difference from the untreated control groups, there was very little difference between the treated groups. In one group, MC 2865 in Exp. 1, the new drug was probably more efficacious than potassium arsenite in the dosages used.

The technical assistance of Virginia Havel is gratefully acknowledged.

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Chemotherapeutic Activity of 6-Sulfanilamido-2,4-Dimethylpyrimidine (Elkosin) (19667)

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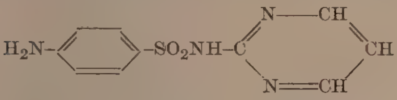
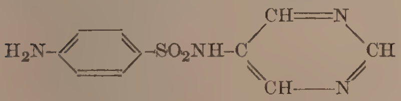
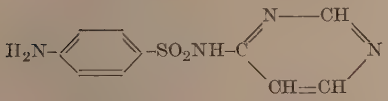
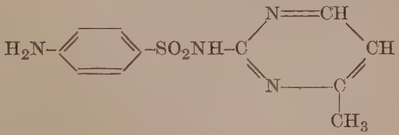
Among the heterocyclic substitutes of sulfanilamide, the sulfanilamidopyrimidines occupy an important place as chemotherapeutic agents because they include several of the most effective and best tolerated sulfonamides known to date. A comparison of the properties of members of this group of compounds clearly demonstrates the marked differences in tolerance and therapeutic activity which take place by slight variations in the substitutions and the attachment of the pyrimidine ring to the sulfanilamide nucleus. 2-Sulfanilamidopyrimidine (sulfadiazine) possesses excellent clinical activity(1) and according to Roblin and coworkers(2,3) it exerts very good protection in experimental mouse infections. On the other hand, the 6-isomer is completely ineffective in the animal body although it exhibits bacteriostatic activity *in vitro* equal to that of sulfadiazine. The 5-isomer was found to have less *in vivo* activity than the 2-isomer.

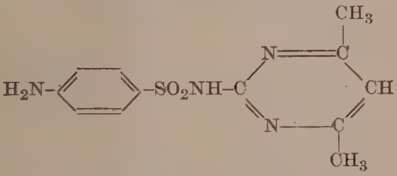

2-Sulfanilamidopyrimidine (Sulfadiazine)


5-Sulfanilamidopyrimidine


6-Sulfanilamidopyrimidine

Mono- and di- substitutions on the pyrimidine ring, especially those with one or two methyl groups, also bring about considerable changes with regard to therapeutic and pharmacologic behavior when compared with the parent, unsubstituted sulfanilamidopyrimidines. Of these substituted compounds, 2-sulfanilamido-4-methylpyrimidine (sulfamerazine) (2), 2-sulfanilamido-4,6-dimethylpyrimidine (sulfamethazine) (3,4) and 6-sulfanilamido-2,4-dimethylpyrimidine (Elkosin) (5-7) are of chemotherapeutic importance.


2-Sulfanilamido-4-methylpyrimidine (sulfamerazine)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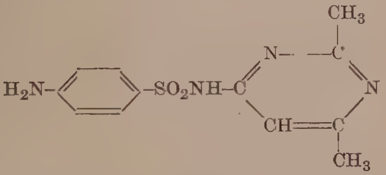
6-Sulfanilamido-2,4-dimethylpyrimidine (Elkosin)

Among the methylated derivatives, the 2,4-dimethyl compound, Elkosin, merits special consideration. According to Meier, Allemann and Meyenburg(5), it is appreciably more soluble in urine over a wide pH range when compared with sulfamethazine and sulfadiazine and has as well, a very low order of acetylation. These workers reported on the broad therapeutic effectiveness of Elkosin in streptococcal and pneumococcal infections in mice, since doses ranging from 0.5 to 24 g/kg administered daily for 6 consecutive days were highly effective.

The results of an experimental study carried out in our laboratory, of the chemotherapeutic properties of Elkosin in a variety of mouse infections are presented in this paper.

TABLE I. Therapeutic Activities of Elkosin, Sulfadiazine and Sulfisoxazole as Indicated by Average Survival Time.

	Avg survival time (days) of infected mice treated with:												
	Elkosin, g/kg				Sulfadiazine, g/kg				Sulfisoxazole, g/kg				Untreated controls
Test organism	5	2.5	1	.5	5	2.5	1	.5	5	2.5	1	.5	
a. Bacilli													
S. pyogenes	21	17.7	15.3	10.2	17.1	17.8	16.2	21	15.6	17.2	16.6	14.9	.4
D. pneumoniae	13	15.2	13.8	6.1	13.1	8.2	16.1	11.5	1.3	10.3	9.3	6	1.4
Cl. tetani	1	.6	.5	1	.5	.7	.6	.7	.6	.8	.8	.7	.6
Kl. pneumoniae	17.3	21	21	21	15.1	13.9	19.5	21	21	20.9	20	17.3	.5
S. typhosa	3.9	7.7	4.2	4.6	3	.8	4.4	5	.5	4.9	6.1	8.2	.5
S. typhi-murium	8	6.1	4	9.1	7.7	8.3	8.9	7.6	8.1	4.3	8.6	4.7	.6
S. schottmuelleri	21	21	18.7	16.7	14.8	21	21	21	18.3	19.3	19.5	19.2	2.2
E. coli	21	20.2	19.5	19.4	13.9	16.4	14.9	18.9	9.6	14.3	21	21	1.6
b. Fungi													
N. asteroides	17.8	18.1	18.1	19.9	9.1	15.9	20	20	6.6	17.7	17.2	13.1	6.2
C. albicans	21	21	—	—	.9	.8	1.2	.9	.6	.8	.8	.7	.8

Methods. Chemotherapeutic tests with CFW mice, each weighing approximately 18 g were performed with the following organisms. *Strep. pyogenes,* strain C203; *Diplococcus pneumoniae,* type 1; *Clostridium tetani; Klebsiella pneumoniae; Salmonella typhosa; Salmonella typhi-murium; Salmonella schottmuelleri; Escherichia coli; Nocardia asteroides* and *Candida albicans.* All animals were *infected intraperitoneally* with 100 to 5000 lethal doses of the various organisms studied. The infecting doses of *Salm. typhosa, Salm. typhi-murium, Salm. schottmuelleri, E. coli, Noc. asteroides* and *Candida albicans* were administered in 4% gastric mucin, prepared according to the method of Runyon(8). For purposes of comparison, sulfadiazine and sulfisoxazole(9) were included in these tests. The latter substance, although not a sulfanilamidopyrimidine, was selected because of its recent introduction for use in urinary tract infections. Suspensions of the test substances were given *per os* one hour before infection. Treatment was continued daily in 2 equally divided doses for 5 days and mice were observed for a total of 21 days. Random subculture of organs from succumbing mice were made in order to verify that death was due to the infecting organisms employed.

Results. A. *Toxicity for mice.* Groups of 10 uninfected mice were given Elkosin, sulfadiazine and sulfisoxazole at daily dose levels of 1.0 and 5.0 g/kg for 5 consecutive days. The drugs were administered *per os* daily in 2 equally divided doses. No deaths occurred as a result of this treatment nor did the mice show any indication of toxic effects. A repetition of this experiment yielded similar results.

B. *Protection tests.* The therapeutic effects of the sulfonamides, shown in Table I, are expressed as "average survival time (days)" per mouse.

Summary. 1. 6-Sulfanilamido-2,4-dimethylpyrimidine (Elkosin) possesses a high degree of effectiveness in experimental mouse infections induced by *Strep. pyogenes, Diplo. pneumoniae* and representative species of the gram negative bacteria. No evidence of protection was observed with *Clos. tetani* and only slight activity was obtained in *Salm. typhosa* infection. 2. Equally favorable results were obtained in infections with *N. asteroides* and *C. albicans.* In the case of the latter infection, Elkosin proved to be highly effective.

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Propagation of Mumps Virus in Suckling Mice and in Mouse Embryo Tissue Cultures. (19668)

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Adaptation of mumps to mice, undertaken to facilitate investigation of this disease, has been accomplished in 2 different ways. In one series of experiments 20 preliminary passages in mouse embryo tissue culture were carried out before virus grew consistently in brains of suckling mice. Later it was found that mumps virus could be more readily adapted to suckling mice by prior passage in suckling hamsters(1). Illness and death occurred only in the latter series of mice, infections being inapparent in those originally inoculated with tissue culture material. It is felt that these investigations have practical interest, as they may pave the way for use of mice in mumps neutralization tests. In addition, mumps infections of mice give some indication of the varied potentialities of this virus as an agent of disease.

Materials and methods. Tissure culture. With modifications, methods employed in tissue culture were ones developed by Earle and associates(2-6). The nutrient fluid consisted of horse serum 40%, balanced saline (Earle's) 40%, and 1:1 extract from 9-day chick embryos, 20%. Antibiotics added to the saline gave it a concentration of 5 mg of streptomycin and 500 units of penicillin per ml. Supporting tissue consisted of mouse embryos in an advanced stage of development. These were comminuted by forcing them through an 18-8 stainless steel sieve of 24 mesh, .014 inch wire diameter(6). Virus containing inocula were mixed directly with tissue pulp and the mixture pipetted into T-60 flasks,* prior to addition of 8 ml of nutrient fluid. Once the cell suspension was evenly distributed over the floor of the flask a sheet of perforated cellophane was dropped on it. Supernatant fluids were drained off and fresh fluid added so as to give a total of 3 fluid changes a week. In later passages, mouse embryo juice was used in the last 2 of these changes. At weekly intervals, the cellular material was scraped from the cellophane, ground, and frozen before using as a virus inoculum for the next tissue culture passage. Roughly 0.5 ml of cellular debris plus fluid were carried over from one passage to another. Precise determination of total dilutions of virus included in the mixture of cells and fluid, was not attempted. Serum ultrafiltrates were assayed in a few alternative passages, but multiplication of virus did not appear to be sustained in this medium. *Virus.* The strain of mumps virus employed for both animal and tissue culture experiments was one originally isolated from human milk(7) and carried through 6 to 7 passages in the amniotic cavity of embryonated eggs. *Mouse passages.* Almost all passages were carried out with A strain mice, although a few regular Swiss mice were employed initially. Suckling mice were inoculated intracerebrally at 1 or 2 days of age and brains were harvested a week later, being ground without abrasive and made up as extracts diluted 1:10 in meat infusion broth.

Identification of virus. Supernatant fluid from each weekly tissue culture passage was inoculated into 7-day embryonated eggs by the amniotic route. Amniotic fluids, harvested separately a week later, were used in hemagglutination tests to determine the presence of mumps virus. Brains harvested from each weekly passage in mice were inoculated in eggs in similar fashion, using a 1:10 dilution. All positive amniotic fluids were pooled and titered for hemagglutinin content. Identification of virus, done periodically in the course of tissue culture and animal passages, was by anti-hemagglutination and complement-fixation tests run with specific immune sera(8). Routine cultures of all materials in whole meat broth and on blood agar slants were used to check on bacterial contamination.

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* The design of this flask will be published later.

Growth of virus in tissue culture. Mumps virus was carried through 20 weekly passages in mouse embryo tissue culture. These passages were continuous except for the third and sixth in which amniotic fluids, from eggs infected with material from the preceding tissue culture passage, were used as inocula. No cellular changes ascribable to virus were observed. Hemagglutinins were not demonstrable in supernatant fluids, although these fluids were infectious for eggs in dilutions as high as 10^{-4}. Attempts were made to grow mumps virus in mouse tissues other than those from embryos. Mammary gland, placenta, liver, and suckling mouse brain were all assayed. In addition a mixture of cells from various adult organs including uterus, spleen, liver, and kidneys was cultured in mouse embryo juice. In these tissues, mumps virus had either disappeared or was only barely demonstrable after 7 days, failing to appear in subsequent passage. Cultures of a mammary adenocarcinoma and a hepatoma of mice were tried with similar results, except that virus seemed to survive particularly well in these tissues. To check the thermo-resistance of mumps virus, sealed ampules of infected amniotic fluids, freshly harvested, were placed in an incubator at 36°C. On 2 separate trials, mumps virus was found to remain infectious after 9 days at this temperature, indicating that virus might passively survive a single tissue culture passage.

Inoculation of mice with tissue culture virus. In the course of the first 10 passages in mouse embryo tissue culture, repeated attempts were made to transmit the virus to suckling and to adult mice, none of which developed signs of illness. Mumps virus as grown in tissue culture did, however, show an unexpected ability to survive in mouse brain, particularly in young suckling mice. Amniotic fluid, from eggs inoculated with 10th passage mouse embryo tissue culture, was inoculated intracerebrally into one-day-old mice in amounts of 0.03 ml. Brains of 3 of these mice, harvested and pooled on the following day, infected eggs when diluted 10^{-5}. Similar brain pools had titers of 10^{-3} and 10^{-2} at 7 and 12 days respectively after inoculation. The original fluid infected eggs in a dilution of 10^{-8}. Continued passage was not successful. After 15 passages in mouse embryo tissue culture, mumps virus was carried through 5 successive transfers in brains of suckling mice. For 3 transfers, brain extracts were readily infectious for eggs, but extracts of the 4th and 5th transfers were markedly less so and those from the 6th passage contained no demonstrable virus. Repetition of this experiment led to similar results. Fifth passage brain extract infected eggs in a dilution of 10^{-2}. Mumps virus carried through 20 tissue culture passages was better adapted to growth in suckling mice, as indicated by 13 successive (*in vivo*) passages. Some loss of egg-adaptation appeared to result from growth in mice. Thus brain extracts from the first 7 mouse passages infected most eggs inoculated, positive amniotic fluids having hemagglutinin titers of 1:320 or better. From the 8th passage on, fewer eggs became infected, the pattern of the hemagglutination was less firm, and positive fluids had low hemagglutination titers. When such fluids were reinoculated into eggs, high hemagglutinin titers were again obtained. No signs of illness were noted in any mice receiving tissue culture virus.

Growth of mumps virus in mouse brain following passage in brains of suckling hamsters. When mumps virus, freshly isolated from a patient in amniotic fluid was used to inoculate suckling hamsters intracerebrally, illness and death usually followed as elsewhere described (1). After 6 hamster passages, the virus was inoculated into suckling mice. For the first 5 mouse brain passages, there was no sign of illness. On 6th passage, however, a majority of mice inoculated developed a striking illness. No 2 mice presented the same clinical picture, but several had spastic convulsions, one dying immediately afterward. Some had arched backs, and others were hyperexcitable, leaping and running around their jars. Although illness was absent in the next 2 passages, it reappeared and persisted in the 9th and following passages. It has been possible to isolate mumps virus from all 19 passages made to date. At present, by harvesting only mice having definite signs of illness, which is usually possible by the 11th day after inoculation, it is hoped to develop a more uniformly fatal strain of virus. Pathological studies, now in progress(9), indicate that, as in suckling hamsters, there are marked histologic changes in the brains of suckling mice having clinical signs as a result of infection with mumps virus.

Discussion. Experiments described above demonstrate that mumps virus will grow readily in mouse embryonic tissue. Weller and Enders(10), who were first to propagate this virus in tissue culture, used amniotic membranes from chicken embryos and a strain of virus which had previously gone through 42 egg passages. They demonstrated production of hemagglutinins in their tissue cultures, whereas hemagglutinins were at no time demonstrable in cultures of mouse embryo tissue. Apparently, this lack of hemagglutinins was not due to the presence of inhibitors, for when supernatant fluids were tested against mumps infected amniotic fluid, no inhibition of hemagglutination occurred. Although supernatant tissue culture fluid infected eggs in a dilution of 10^{-4} this relatively low titer of virus might account for lack of hemagglutination. To our knowledge, however, hemagglutinins have not been demonstrated with mumps virus propagated in mammalian tissue.

In initial attempts at adaptation, passage of mumps virus in mouse brain was not initially successful, either with infected amniotic fluid or with supernatant fluid from the first 10 tissue culture passages. Ability of mumps virus to survive for 12 days in suckling mouse brain suggested, however, that with further propagation in mouse embryo tissue culture, mouse adaptation might be achieved.

Adaptation to growth in suckling mice developed progressively as virus from the 10th, 15th and 20th passage tissue cultures was assayed. Although tissue culture led to a strain of mumps virus which would multiply consistently in suckling mouse brain, this strain has failed so far to develop obvious pathogenicity. This lack of virulence may reflect the fact that the mouse embryos, cultivated in their entirety, consisted of many tissues. Mumps virus propagated in such media would not, necessarily, develop specific affinity for brain tissue, as encountered in *in vivo* transfers. When a strain of mumps which had caused illness and death in suckling hamsters was used in mice, pathogenicity, not encountered initially, appeared suddenly in the 6th passage. It became permanently established when mice were carefully selected for harvesting. In reviewing these experiments, it seems possible that tissue culture actually attenuated the intracerebral virulence of mumps virus for mice. Mumps virus as freshly isolated from its human host is naturally virulent for suckling hamsters(1) and apparently this natural virulence can be carried over into mice. Another point of interest is that ability of virus to multiply in brain tissue seemed to have no relation to its pathogenic effects. Thus in suckling mice originally inoculated with hamster brain containing virus and developing severe illness, the amount of virus recoverable in eggs appeared no greater than in those mice of the tissue culture series which never became ill.

As mumps virus from any source was progressively passed in mouse brain it appeared to lose much of its adaptation to embryonated eggs, as indicated by the lesser number of eggs becoming infected and the lowered hemagglutinin titer of infected amniotic fluids. Second egg passage, however, would fully restore high hemagglutinin titers. Although factors may have been involved other than loss of egg adaptation, these results suggest that mumps virus, when propagated for a sufficient time in mouse brain, resembles more closely the virus as found in man. Thus in the course of making virus isolation from human saliva and spinal fluids(11), it was observed that although little or no hemagglutination appeared in amniotic fluids on first egg passage, such fluids would become strongly positive on 2nd and 3rd egg passages.

In continuing experiments, efforts are being made to develop a variant of mumps virus which will be more uniformly fatal for suckling, and if possible, for adult mice. A development of such virulence, if it could be achieved, should make it possible to perform neutralization tests in mice. As described above such tests are already under study.

Summary. 1. Mumps virus has been carried through 20 passages in mouse embryo tissue culture in the course of which it developed a progressive ability to grow in brains of suckling mice, without apparent illness of the animals. 2. Another strain of mumps, after passage in brains of suckling hamsters, also multiplied readily in the brains of suckling mice, leading to illness and, in a small proportion, to death on continued passage. 3. Practical and theoretical implications of this adaptation of mumps virus to mice have been discussed.

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Occurrence of Coxsackie Virus Complement Fixing Antibodies in Sera of Normal Monkeys.* (19669)

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In the course of investigations regarding Coxsackie virus complement fixing (cif.) antibodies in monkeys, it was found that a homologous hyperimmune Leon type poliomyelitis monkey serum manifested a high titer (1:64) for one of the Coxsackie virus (C virus) prototype antibodies (Easton-10)(1). The possible reasons for its presence in a specific poliomyelitis monkey serum seemed to warrant additional experimentation.

Materials and methods. Sera. Homologous hyperimmune monkey sera were employed as follows: the Petry strain (Leon type poliomyelitis) and the Brunhilde strain were received from Dr. Jonas Salk; Ilda and Stoddard strains (Brunhilde types), as well as MV and MEF1 strains (Lansing types) were received from Dr. Louis Gebhardt; and the Lansing strain from Dr. Robert Ward. Hyperimmune monkey and mouse sera prepared in this laboratory for the FA strain of murine encephalomyelitis virus were also employed, as were sera from monkeys hyperimmunized with the Texas-1 type of Coxsackie virus (Monkey 4197) using infant mouse brain as immunizing agent, and with the Easton-2 (Dalldorf's type 1) Coxsackie virus (Monkey 4766) using muscle-bone suspensions from infected infant mice as antigen. Specific hyperimmune Coxsackie virus mouse sera were prepared for all types of antigens used in the c.f. tests (see below)(2). Sera were obtained from 19 rhesus (*Macaca mulatta*) and 15 cynomolgus (*Macaca irus*) monkeys which had been in the laboratory for periods of time varying from 1 to 43 days. They had been uninoculated prior to bleeding. Ten of the rhesus monkeys were bled at 4- to 7-day

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* Aided by a grant from The National Foundation for Infantile Paralysis.

intervals for 23 days. Also two pools of serum from a number of uninoculated rhesus and cynomolgus monkeys were used.

Complement fixing antigens. Ten Coxsackie virus types were employed in the majority of the complement fixation tests. They were: Easton-10 (E-10), Easton-14 (E-14), Dalldorf's type 2 (D-2), Dalldorf's type 3 (D-3), Alaska-8 (Alaska), Texas-1, Easton-2 (E-2 Dalldorf's type 1), Nancy, Connecticut-5 (Conn-5), Ohio-1. Complement fixation tests with antigens of 5 additional types were performed on the sera of 5 of the normal rhesus monkeys, the latter types including Texas-12, Texas-13, Texas-14, Texas-15, and Israel-7(1). The description of the preparation of the antigens (20% protamine treated muscle-bone suspensions) has been reported(3). Concentrated purified antigens were prepared by ultracentrifugation for the E-10, D-3, and Texas-1 viruses according to the method described previously(4). The antigen of FA murine encephalomyelitis virus was the supernate resulting from the centrifugation at 18,000 r.p.m. for 30 minutes (International PR-1 centrifuge) of a 10% suspension of infected mouse brains. That of the infant mouse-adapted MEF1 strain of poliomyelitis virus of Casals and Olitsky(5) was the supernate of a 50% suspension of infected 3-day-old mouse brains centrifuged at 4°C at 18,000 r.p.m. for 1 hour. Normal antigen was prepared in like manner from uninoculated mice of the same age. *Complement fixation test.* The c.f. titers reported below were obtained by means of the 50% hemolysis method according to Wadsworth(6). Many of the results were checked by the plate c.f. test of Fulton and Dumbell(7,3).† There were no significant differences in the results obtained with the two methods.

Temperature of inactivation. For routine use in the c.f. tests the sera were inactivated at 60°C for 20 minutes. A number of sera (see below) were also heated for 20 minutes at the following temperatures in degrees C: 63, 65, 68, 70, 73, 75. All sera were diluted 1:4 before heating. The deviation of the water bath at each temperature was ±0.1°C. *Neutralization tests.* These were carried out according to the method used in this laboratory(2) employing 100 ID_{50} of virus against constant or varying dilutions of the sera. In addition tests employing varying dilutions of the viruses with constant serum dilutions were also performed. For the neutralization tests all sera were inactivated at 56°C for 30 minutes, specific neutralizing antibodies being stable at this temperature(2). *Serum absorptions.* Serum from monkeys 4197, 4766, and 5782 (a normal rhesus monkey) was subjected to the following absorption procedures: 1) Sheep cell absorptions were performed by adding to 0.8 ml of undiluted serum 3.2 ml of centrifugally packed sheep cells which had been washed three times with physiological saline. The mixture was allowed to remain at room temperature with occasional shaking for one hour after which it was centrifuged and the supernatant fluid used for the c.f. antibody determinations. 2. Absorption by boiled guinea pig kidney and boiled beef cells were done according to the methods described by Kolmer and Boerner(8). In each case the serum was heated at 60°C for 20 minutes at the end of the absorption period.

Lyophilization and chloroform extraction. Lyophilization of sera from immunized monkeys 4197 and 4766, and from normal monkey 5782, as well as extraction of the lyophilized samples with chloroform, was carried out. Three volumes of chloroform were added to each sample and removed after standing 15 minutes at room temperature with occasional shaking. This was repeated 3 times making a total extraction volume of 9 times. Reconstitution in distilled water of lyophilized and chloroform extracted sera took place at room temperature for ½ hour after which they were heated at 60°C for 20 minutes. *Dialysis.* One serum (normal rhesus 5789) was dialyzed overnight at room temperature in 1000 volumes of distilled water before being used in the c.f. test.

Results. The c.f. titers obtained with Coxsackie, FA, and MEF1 antigens in various homologous hyperimmune monkey sera are presented in Table I. The reactions between

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† The help of Dr. Guillermo Contreras is gratefully acknowledged.

TABLE I. Coxsackie Virus c.f. Antibody Titers of Some Specific Homologous Hyperimmune Monkeys and Mouse Sera.*

Sera from monkeys immunized with:	Source	Reciprocals of c.f. titers with following antigens† E-10	E-14	D-2	D-3	Alaska	Texas-1	E-2	Nancy	Conn-5	Ohio-1	MEF1	Normal	FA
Brunhilde	Pittsburgh	64	48	64	48	64	16	0	0	0	0	0	0	0
Ilda (Brun.)	Utah	32	48	24	16	48	0	0	0	0	0	0	0	0
Stoddard (Brun.)	"	6	0	8	0	0	0	0	0	0	0			0
Leon	Pittsburgh	96	32	96	32	4	6	0	0	0	0	0	0	0
MEF1 (Lansing)	Utah	4	0	0	0	0	0	0	0	0	0	128	0	0
MV (")	"	12	12	24	6	12	6	0	0	0	0	64	0	0
Lansing	New York	64	12	48	0	64	48	0	0	0	0	256	0	0
FA (monkey)	Yale	12	12	24	0	24	24	0	0	0	0	0	0	48
FA (mouse)	"	0	0	0	0	0	0	0	0	0	0	0	0	96

0 = titer <1:4.

* Sera obtained from 4 different laboratories.

† See text for explanation of antigens.

TABLE II. Numbers of Normal Monkeys with c.f. Reactions at 1:4 or Higher Serum Dilution to Indicated Virus Types.†

No. of monkeys	Days after arrival in laboratory	No. of monkeys with c.f. reactions to: E-10	E-14	D-2	D-3	Alaska	Texas-1	E-2, Nancy, Conn-5, Ohio-1 types*	No. of monkeys with no c.f. reactions
6 R*	1	3	5	1	0	0	0	0	1
6 C	7	4	3	3	2	3	1	0	1
3 R	12	2	1	1	0	0	0	0	1
10 R	21	5	6	4	5	1	1	0	4
9 C	43	8	6	6	6	8	1	0	1
Total 34		22	21	15	13	12	3	0	8

* R = rhesus. C = cynomolgus. 0 = titer <1:4.

† Pooled rhesus and cynomolgus sera from other monkeys had c.f. reactions to all 6 types. None of the normal sera reacted with E-2, Nancy, Conn-5, Ohio-1, c.f. antigens.

the Coxsackie virus antigens and mouse hyperimmune sera were specific(1,3), no cross reactions being encountered. Omitted from Table I are data indicating that none of the Coxsackie virus mouse antisera reacted with the MEF1 or FA antigens.

The titers obtained with normal rhesus and cynomolgus monkey sera are similar in every respect to those found in the sera of Table I. Considering titers of 1:4 or higher as positive reactions, Table II indicates the number of normal monkeys, as well as the 2 pools of normal sera, manifesting the Coxsackie virus c.f. antibodies.

Both tables show that when reactions between monkey sera and antigens were seen, they were confined for non-Coxsackie antigens to the homologous reaction (FA and MEF1 in Table I) and to only 6 of the 10 Coxsackie types tested, *viz.* E-10, E-14, D-2, D-3, Alaska, Texas-1. All 6, however, were not present at the same time in every animal. Thus 8 of

TABLE III. Effect of Heat and Dialysis on c.f. Titers in Normal Monkey Serum.

Monkey	Serum treatment		Reciprocals of c.f. titers:† E-10	E-14	D-2	D-3
5789	Dialysis* and heat	60	12	112	24	48
		65	0	24	6	4
		68	0	20	0	0
		70	0	6	0	0
5782	Heat	60	32	32	16	32
		63	24	16	16	24
		65	8	12	8	16
		68	0	0	0	8
		70	0	0	0	0

* See text. Results were similar before dialysis.

† Neither monkey had c.f. antibodies to Alaska, Texas-1, E-2, Nancy, Conn-5, Ohio-1.

0 = titer <1:4.

TABLE IV. Effect of Heat on c.f. Titers in Monkeys Hyperimmunized with Coxsackie Viruses.*

Monkey and immune status	°C	Reciprocals of c.f. titers: E-10	E-14	D-2	D-3	Alaska	Texas-1	E-2	Nancy	Conn-5	Ohio-1
4766											
Pre-immunization	60	8	6	8	6	0	0	0	0	0	0
Immunized with E-2	60	48	64	48	80	56	32	160	48	0	0
	63	16	48	16	64	32	12	96	14		
	65	8	24	8	32	24	8	24	10		
	68	4	12	4	16	8	0	16	4		
	70	0	8	0	12	6	0	12	0		
	73	0	0	0	4	0	0	4	0		
4197											
Pre-immunization	60	0	64	0	48	48	0	0	0	0	0
Immunized with Texas-1	60	64	160	80	64	80	160	0	0	0	0
	63	32	96	20	32	32	128				
	65	24	32	8	12	20	80				
	68	16	8	0	0	6	32				
	70	6	6	0	0	0	16				
	73	0	0	0	0	0	6				
	75	0	0	0	0	0	0				

* c.f. titers of the immune sera after various additional treatments (see text) were not significantly different from those before treatment.

0 = titer <1:4.

34 monkeys had none of the 10 antibodies (Table II), whereas 26 had some combination of the 6 previously mentioned types. When titrated, the serum values ranged from 1:4 to 1:128; most were in the neighborhood of 1:32. Table I indicates that 6 of 8 hyperimmunized monkeys (poliomyelitis and FA immune) also manifested them in high titers. Sera from the latter had been prepared in 4 separate laboratories, although this appears to be of little consequence in view of the reaction taking place in normal monkey sera.

In a series of 10 monkeys bled at 4 to 7 day intervals for a period of 23 days, none showed any significant change in Coxsackie virus c.f. titers for that time. Four of these animals never had any of the 10 antibodies even though 2 of them were cage-mates of 2 others which did have some of them. Five of these 10 monkeys were tested with 5 "new" Coxsackie virus types, Texas-12, Texas-13, Texas-14, Texas-15, and Israel-7(1). The antibodies were distributed as follows:

No. of animals	No. of 10 "old" type antibodies present	No. of 5 "new" type antibodies present
3	0	0
1	4	2
1	6	5

Tables III and IV present the c.f. titers resulting when sera from uninoculated and Coxsackie virus hyperimmune monkeys respectively, were subjected to the indicated treatments. Increasing heat of inactivation was the only agent which affected the titers significantly. In hyperimmune monkey 4197 (Table IV), although the only virus given was Texas-1, the E-10, D-2, and Texas-1 c.f. antibodies rose significantly as a result of hyperimmunization whereas in monkey 4766 (Table IV), hyperimmunized with E-2 virus, all antibodies except Ohio-1 and Conn-5 rose significantly or appeared as the result of hyperimmunization.

Tests for *neutralizing* antibodies in Monkeys 4197 and 4766 indicated that only those for Texas-1 in the former, and those for E-2 in the latter, were present after hyperimmunization. They had been absent prior to immunization. *Neutralizing* antibodies for Coxsackie viruses were not detected in any of the normal monkeys nor in the monkeys hyperimmunized with FA virus or the poliomyelitis viruses.

Discussion. From the results presented it seems clear that both rhesus and cynomolgus monkeys, as they arrive in the laboratory, may possess substances in their sera which are capable of reacting like complement fixing antibodies to certain of the Coxsackie viruses, and that, as far as has been determined, they are not accompanied by heat stable neutralizing antibodies. The striking pattern of the reactions, always being confined to antibodies of from one to 6 virus types of 10 types tested is noteworthy. (Five additional types were tested and antibodies to all were found.) The occurrence of these in normal monkeys explains their presence in the sera of monkeys immunized with poliomyelitis or other heterologous viruses.

Regardless of whether they were found naturally or produced by hyperimmunization of monkeys, these antibodies could not be differentiated by any of the treatments employed, *viz*. absorption by sheep cells, boiled guinea pig kidney, boiled beef cells; lyophilization with or without chloroform extraction; dialysis; the use of ultracentrifugally concentrated, purified antigens in the c.f. test; or increasing heat of inactivation. Only the latter treatment affected them significantly, but it affected both the natural and specifically induced antibodies in identical fashion.

Absorption of specific c.f. antibodies from monkey hyperimmune sera has not yet been accomplished although it has been repeatedly attempted. Be that as it may, the presence of these substances in monkey sera could conceivably reflect possible subclinical infection in them with agents (including additional Coxsackie virus types) which have antigenic relationships with the 6 (or 11) "reacting" types concerned in this report. It may also be that in the pathological processes resulting in the infected mouse tissues, certain Coxsackie viruses cause substances to be formed which are capable of reacting in the complement fixation test with antibody-like material in the serum of some monkeys. Even so, the question of the relationship between these serum substances normally present in monkeys and specifically induced antibodies remains open, since hyperimmunization with specific Coxsackie virus causes the appearance of, or significant rises in, heterologous Coxsackie virus c.f. antibodies, just as infection with a specific Coxsackie virus does in man(9).

Summary. In the sera of normal rhesus and cynomolgus monkeys substances have been encountered which are capable of fixing complement in the presence of as many as 6 (or 11) antigenically distinct Coxsackie, or C, virus antigens. "Antibodies" to 4 C virus types were consistently absent. The behavior of these antibodies did not differ significantly from Coxsackie virus complement fixing antibodies produced by specific immunization. Accompanying heat stable neutralizing antibodies have not been demonstrated in normal monkeys but only in those specifically immunized.

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Effect of Nitrogen Mustard on Nucleic Acid Metabolism.* (19670)

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Histological, biological and biochemical evidence(1) has been interpreted to implicate nucleic acid metabolism as a possible locus of action of nitrogen mustards. Studies in the rat have demonstrated a decrease in the incorporation of P^{32} into nucleic acids under the influence of the drug(2), while C^{14}-formate incorporation into nucleic acids of the mouse is also depressed(3). Since there was evidence that formate was incorporated into the 4-amino-5-imidazole-carboxamide ribotide (4,5) and that adenine was incorporated into nucleic acids without opening of the ring at the 2 position(6)‡ the hypothesis that free adenine enters the pathway of nucleic acid synthesis after the carboxamide ribotide step appeared logical. On this basis, if a drug affected any of the early steps in the synthesis of the purine ring, a decrease in the incorporation of a simple precursor such as formate would not be associated with a similar decrease in the more complex molecule adenine when both precursors were administered simultaneously. Indeed, aminopterin has been shown to produce a differential effect on formate incorporation compared to adenine incorporation and the problems involved in the experiment have been discussed(7). However, in the following experiment, nitrogen mustard produced similar decreases in the incorporation of N^{15}-adenine and of C^{14}-formate into the adenine of DNA (desoxypentose nucleic acid) of the small intestine of the rat.

Material and methods. Two groups of 10 Sherman strain 200 g rats were injected by intraperitoneal route simultaneously with isotopic formate solution§ (0.014 mM per kilo or 7.6 x 10^7 counts per minute per kilo as determined by gas flow counter), and N^{15}-adenine (4.83 atom % excess N^{15} and in the 1 and 3 positions(8) made up in solution with 2 meq. of lactic acid per meq. adenine and administered 0.2 mM per kilo). Fifteen minutes prior to the administration of the isotopic precursors, the rats of one group each received a single intravenous injection of nitrogen mustard (bis(2 chloroethyl) methylamine hydrochloride, 0.4 mg per ml in 0.9% NaCl, dose 4 mg per kilo), which is approximately 2 to 4 x the LD50 dose(1).

All animals were sacrificed 24 hours after injection and the tissues immediately frozen. Free bases of DNA of small intestine and PNA (pentose-nucleic acid) of small intestine and liver were separated by a column chromatographic procedure and their isotopic levels determined as described elsewhere(7).

Results and discussion. The results of the isotope determinations are presented in Table I. Nitrogen mustard does not appear to have a differential effect on the level of C^{14}-formate as compared to that of N^{15}-adenine. The change in the C^{14} activity in the adenine of the DNA of small intestine was 58%, while the change in N^{15} due to the drug was 75%. Thus the parallel depression of both precursors suggests that nitrogen mustard does not affect specifically any of the steps leading to the synthesis of the purine skeleton.

The second point of interest is the preferential effect of nitrogen mustard on the incorporation of the isotopes into DNA as compared to PNA. Among the purine bases isolated from PNA of liver and intestine, the liver adenine fraction was the only one which showed any decrease in isotope level following drug administration. These results corroborate other evidence which suggests a preferential effect of nitrogen mustard on

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§ Obtained from the Oak Ridge National Laboratory.

TABLE I. Effect of Nitrogen Mustard on Incorporation of C^{14}-Formate and N^{15}-Adenine into Nucleic Acids of Intestine and Liver.

		C^{14} activity, c.p.m./μM			N^{15} atom % excess		
		Control	Mustard	% change	Control	Mustard	% change
			Intestine				
DNA	Adenine	182	77	–58	.089	.022	–75
	Guanine	464	175	–62	.024	.004	–83
	Thymine	461	195	–58			
	Cytosine	10	11	—			
PNA	Adenine	256	259	0	.139	.153	+10
	Guanine	329	330	0	.035	.047	+34
	Uracil	15	14	—			
	Cytosine	9	14	—			
			Liver				
PNA	Adenine	57	47	–18	.139	.127	– 9
	Guanine	25	39	+56	.041	.044	+ 7
	Uracil	9	8	—			
	Cytosine	7	12	—			

DNA, as opposed to PNA metabolism. When P^{32}-phosphate was used as a precursor of the nucleic acid of bone marrow, spleen and thymus, the depression of incorporation into DNA was more marked than into PNA(2) following mustard treatment. The tissue content of DNA was decreased to a greater extent than that of PNA by nitrogen mustard in both the amblystoma embryo(9) and *Escherichia coli*(10). Finally, viruses with a high DNA content were more easily inactivated by nitrogen mustard than viruses with a high PNA content, while pneumococcus transforming factor, a DNA preparation, showed extreme sensitivity(11).

It should be emphasized that the biological and chemical evidence which implicates nucleic acid metabolism (particularly DNA) as the locus of action of nitrogen mustards *in vivo* is indirect. Knowledge regarding reduplication of particles or cells is as yet so meager that such *in vivo* experiments cannot distinguish between a specific effect of the drug on nucleic acid metabolism and an effect of the drug on other portions of the system involved in reduplication.

Summary. 1. The effect of nitrogen mustard on the incorporation of C^{14}-formate and N^{15}-adenine into the bases of PNA and DNA of small intestine and into the bases of PNA of liver of the rat has been investigated. 2. The drug decreased the incorporation of both precursors into adenine of DNA of intestine to approximately the same extent. This suggests that the drug does not affect specifically the synthesis of the purine skeleton. 3. The drug led to a marked decrease of isotope levels in the bases of DNA of small intestine, but little or no change in the levels in PNA bases of both small intestine and liver.

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Effect of Acetylcholine on St. Louis Encephalitis Infection in the Mouse.* (19671)

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It is generally assumed that viruses which multiply only within cells of a particular type must find within these cells a peculiarly favorable metabolic milieu. The most striking examples of viral cytotropism are probably those neurotropic viruses which multiply within neurons. It is not improbable that the enzymatic peculiarities which are responsible for cytotropism may in some instances be related to the physiological function of the host cells involved. In the case of the neurons, a metabolic entity of unique functional importance is the acetylcholine-acetylcholinesterase-choline acetylase system(1,2). For these reasons, it seemed possible that among the many chemical agents which have been shown to modify acetylcholine (ACh) metabolism, some might be found which would alter the course of neurotropic viral infections. In this report the effect of acetylcholine itself on St. Louis encephalitis in mice will be presented.

Material and methods. The strain of St. Louis encephalitis used had been maintained for several years in the laboratory of Dr. G. O. Broun, of the Department of Internal Medicine. The mice, which were of the "Swiss" strain, were obtained from a commercial breeder, and maintained on the Rockland diet, with water *ad lib*. They were of fairly uniform size; those used in the first experiment averaged approximately 20 g in weight, those in the second experiment about 16 g, and those in the third and fourth about 18 g. It had been determined previously that ACh, given intraperitoneally at dosage levels of about 100 mg/kg was well tolerated, resulting only in apparent discomfort (increased salivation and decreased activity) for a period of 5 minutes or less. When doses of 150 mg/kg were given, however, occasional fatalities occurred within a few minutes.

Experimental. In the first experiment, 40 female mice were inoculated intranasally, without anesthesia, with 0.03 cc of a 1% aqueous suspension of brain tissue from a mouse dying of St. Louis encephalitis. The inoculum was given from a suitable calibrated syringe fitted with a 26-gauge needle, and was divided equally between the 2 nares. Five to 10 minutes before inoculation, the 20 mice in the treated group were injected intraperitoneally with 2 mg of ACh in 0.1 cc of distilled water. Similar injections were given daily until death occurred, or for 15 days in surviving animals. The control animals were not treated in any way, since it seemed improbable that the minute amount of distilled water given intraperitoneally could influence the course of the infection. (On the third day after inoculation, one control mouse was accidentally killed in closing a cage door). The second experiment was a repetition of the first with the following changes: The number of treated animals was 40 and the number of controls was 41. The dosage of ACh was 2 mg twice a day for the first 3 days; thereafter it was 1.6 mg/mouse daily. The third experiment was a repetition of the second except for the number of animals involved and the fact that the mice were of mixed sexes. The fourth experiment was a repetition of the second with the following difference: The first injection of ACh was given 18 hours before virus inoculation and the second injection 18 hours after virus inoculation.

Results. The figures given in Table I show that in all 4 experiments the survival rate in the treated animals was significantly higher than that in the controls. The probability that the figures representing totals for control and treated animals are significant, as determined by the χ^2 method(3) is approximately 6000:1. With rare exceptions, animals which lived did not at any time show evidence of

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TABLE I. Effect of Acetylcholine on St. Louis Encephalitis in Mice.

	No. of mice	Died	Lived	% survival
Control	19	10	9	47
ACh	20	4	16	80
Control	41	29	12	29
ACh	40	16	24	58
Control	55	40	15	27
ACh	50	27	23	46
Control	48	34	14	29
ACh	42	21	21	50
Total controls	163	113	50	31
Total ACh	152	68	84	55

illness, and animals which became ill died within 24-48 hours. The great majority of deaths occurred on the 6th and 7th days after inoculation; rarely an animal became sick as late as the 11th day and died on the 12th or 13th day. The average survival time of the treated mice which died was about 24 hours longer than that of the control mice which died.

Discussion. In the first 3 experiments, although virus was never injected until the period of increased salivation had apparently ended, the possibility existed that persistent changes in the nasal mucosa might have resulted in increased local inactivation of the virus. For this reason, in the fourth experiment the first and second intraperitoneal injections of ACh were given 18 hours before and 18 hours after the introduction of the virus into the nares. Since the increase in survival rate of the treated mice in this experiment was comparable to that in the other experiments, it is believed that the possible importance of local changes has been fairly well excluded, particularly since it has been shown (4) that no virus can be recovered from minced nasal mucosa 4 hours after the intranasal instillation of 10 times the virus dosage used in our experiments.

The mechanism of action of ACh in reducing the mortality from this viral infection can be determined only by further work. There is evidence that ACh, when present in high concentrations, may increase the rate of synthesis of ACh *in vitro*(5). The ACh-cholinesterase system is apparently an important factor in determining the permeability of certain cells(2). Cholinesterase inhibitors have been shown to influence dehydrogenase systems in the brain(6), suggesting that cholinesterase may have important effects on the general metabolism of neurons.

It is fortunate that a large group of carefully studied agents capable of inhibiting cholinesterase are available for further study. These include physostigmine, prostigmine, certain bacterial exotoxins, cobra venom, curare, caffeine, acetyl-B-methylcholine, the alkyl fluorophosphonates and many others. The choline acetylase system also has been extensively studied from the viewpoint of inhibition and stimulation(2). It seems possible that the use of these agents may make it possible to relate the multiplication of certain neurotropic viruses to specific metabolic entities within the neurons which they invade.

Summary and conclusions. Mice inoculated intranasally with the virus of St. Louis encephalitis were treated by the intraperitoneal injection of acetylcholine in large but well tolerated dosages. The survival rate of the treated animals was 55%, as compared to 31% for the control animals. Reasons are given for believing that the observed effect may be the result of alterations in cerebral metabolism.

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Augmentation of Rat Ovarian Weight Induced by Chorionic Hormone.*

(19672)

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The preliminary administration of either estrogenic hormone(1,2) or testosterone(3) to immature female rats results in an augmentation of the ovarian weight induced by the chorionic hormone. In the present study a number of drugs and endocrine substances were investigated for possible similar "augmentation" properties.

The rats employed were of the Sprague-Dawley strain, and were 21 days of age at the beginning of each experiment. On day 1 3 subcutaneous injections of the material under investigation were given, and on day 2 3 injections of 0.25 cc (250 I.U.) of chorionic hormone (A.P.L.-Ayerst) were administered intraperitoneally. The rats were sacrificed on day 6 when body, uterine and ovarian weights were recorded. The ovaries were fixed in Bouin's solution for section and microscopic examination.

A total of 108 rats were employed in 2 series of experiments, and a different batch of chorionic hormone was used in each case. The results of the first series are shown in Table I, and it is seen that a slight augmentation of the ovarian weights over those of the controls receiving plain water was noted in the case of Padutin-Winthrop (insulin-free hormone from the pancreatin gland), Acthar-Armour (ACTH), Cortone-Merck (Cortisone), and Pitressin tannate in Oil (P.D.Co.). No augmentation was seen with Priscol-Ciba (benzazoline hydrochloride) nor Bistrium Bromide-Squibb (trimethylammonium bromide). The ovaries of the rats given neostigmine (Prostigmine-La Roche), however, weighed .042 and .049 g, representing a 27 and 48% augmentation over those of the water controls.

The results of the second series of experiments are illustrated in graphic form in Fig. 1, each column representing the average ovarian weights from groups of 5 or 6 rats. The first column (a) shows .037 g as the ovarian weight resulting from giving 750 I.U. of chorionic hormone on day 2, but no injection on the first day. The animals of columns (b), (c), and (d) received sesame

TABLE I. Chorionic Hormone 750 I.U. Given Intraperitoneally on Day 2 to Each Group.

Day 1 (3 subcut. inj.) Total dose	No. of animals	Avg body wt, g	Uterus, g	Ovaries, g
Water, 1.5 cc	4	67	.146	.035
	4	66	.146	.029
	3	60	.147	.034
Priscol, 18.75 mg	5	58	.134	.029
	4	63	.127	.032
Bistrium, 7.5 mg	5	62	.137	.024
	4	67	.133	.038
Padutin, 7.5 u	5	66	.143	.043
	4	63	.124	.037
ACTH, 1 mg	8	65	.144	.038
Cortone, 7.5 mg	3	59	.131	.038
Pit. tannate in oil, 3.75 pressor u	6	68	.121	.034
Neostigmine, .075 mg	4	63	.143	.042
	3	60	.110	.049

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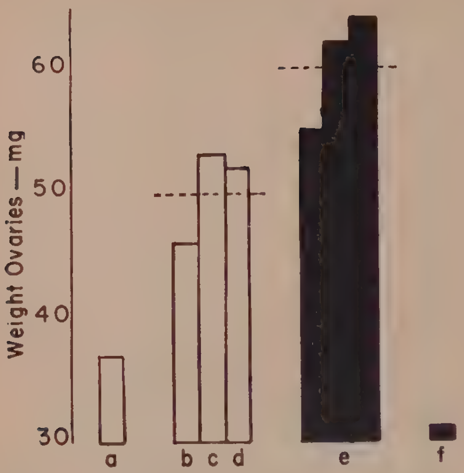

FIG. 1. Rats of column (a) received 750 I.U. of chorionic hormone without a preliminary injection. Rats of succeeding columns received, on day 1, sesame oil (b), cottonseed oil (c), plain water (d), and neostigmine (e), while on day 2 they were given the chorionic hormone. The order was reversed in those of (f), that is, day 1 the chorionic hormone and day 2 the neostigmine.

oil (0.75 cc), cottonseed oil (0.75 cc), and plain water (1.5 cc) respectively on day 1, and their average ovarian weight was 0.50 g. Column (e) is of 3 groups of rats given neostigmine (.075 g in 1.5 cc water) on the first day and shows an average ovarian weight of .060 g, an augmentation of 20% over the controls given injections of inert material on day 1 and of 62% over those not given any preliminary treatment.

In a previous report(2) it was shown that if the order in which the hormones were administered was reversed, namely, the chorionic hormone on the first day and stilbestrol on the second, there was no augmentation of ovarian weight. The same is true of neostigmine (Fig. 1f) for ovaries of only .032 g were found when it was given on day 2 and the chorionic hormone on day 1.

No histologic differences from the changes normally resulting from the administration of chorionic hormone were noted in any of the experiments.

Summary. The preliminary administration of various drugs, hormones, and inert substances such as plain oil or water, may result in a slight augmentation of the ovarian weight induced by chorionic hormone. A striking augmentation, however, has been obtained consistently by the injection of neostigmine on the day before giving the chorionic hormone.

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Effect of Various Plasma Volume Expanders on Erythrocyte Size. (19673)

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Quantitative determinations of acute blood volume changes may be made by studying the deviation from the expected biological decay curve of circulating erythrocytes tagged with P^{32}. In the course of studies following intravenous administration of 6% Dextran, one of us (LMM) observed that blood volume increase estimated in this manner was regularly greater than the increase calculated from the change in the peripheral hematocrit(1). This difference might be reconciled if a change in erythrocyte size or shape could be demonstrated. To determine whether such a change could be induced by Dextran or other plasma volume expanders, an *in vitro* experiment was performed under conditions approaching those *in vivo*.

Method. Venous blood was added to an appropriate amount of "balanced" oxalate, then divided into 4 equal aliquots, each of which was placed in a 125 cc siliconized flask. Because it was estimated that when 500 cc of

TABLE I. Hematocrit determinations and observations of crenation and hemolysis at 0, 30, 60, 120 and 240 min. after addition of isotonic saline or one of the expanders to whole blood. Data are derived from studies on 2 subjects.

	Hematocrit					Crenation		Hemolysis	
Time (min.)	0	30	60	120	240	0	240	0	240
Whole blood undiluted Pt. #1	51	49.5	49	50.5	50.5	0	0	0	3+
Saline & Blood 1:10 Pt. #1	45	45	44.5	44	45.5	0	0	0	+
Dextran & Blood 1:10 Pt. #1	44.5	44	44	44	45.5	0	0	0	+
PVP & blood 1:10 Pt. #1	45.5	45	45	45.5	45	0	0	0	+
Plasma & Blood 1:10 Pt. #2	37.5	36.5	36.5	37	37	0	0	0	+
Gelatin & Blood 1:10 Pt. #2	37	36.5	36	36.5	36	0	0	0	+
Albumin & Blood 1:10 Pt. #2	37	36	36	36.5	36	0	0	0	0

a plasma volume expander was administered to an average individual the expander was diluted about 1:10 with blood, the substance under study or normal saline solution was added in this proportion to each of three of the flasks. Whole blood in the fourth flask was kept as a control. All samples were suspended in a constant temperature water bath at 37°C, where they were agitated by a wrist action shaker. Using a small pump, humidified air was bubbled through the blood in each flask. Positive pressure was maintained by use of an outlet vent controlled by a thumb screw, thus minimizing foaming. Samples were withdrawn at 0, 30, 60, 120 and 240 minutes and spun in Wintrobe hematocrit tubes at 3000 rpm for thirty minutes. Erythrocytes were examined microscopically for evidence of crenation. Supernatant plasma was inspected for the presence of hemolysis.

Results. Studies were made on each of the following substances: human plasma (10 cases); 5% serum albumin in isotonic saline solution (10 cases); 6% Dextran (10 cases); 3.5% polyvinyl pyrrolidone in isotonic saline (10 cases); 5% gelatin in isotonic saline (6 cases); isotonic saline (18 cases).

In each instance, hematocrit determinations immediately following addition of the expander to blood showed a value which reflected only dilution. No differences were noted among the expanders, or between them and isotonic saline solution. Within technical limits, hematocrit determinations on the samples from each flask remained unchanged over the four hour period of observation. Minimal crenation was observed infrequently at the end of four hours. As a rule, a trace of hemolysis was noted grossly in the supernatant plasma in the hematocrit tube at the end of 4 hours. This was usually most marked in the whole blood sample. The accompanying table is representative of the results obtained.

Conclusions. Dextran, polyvinyl pyrrolidone, serum albumin, gelatin, plasma and normal saline have no effect on erythrocyte size *in vitro* under conditions simulating those in the body.

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Effect of Cobalt^{60} Gamma Radiation on Susceptibility and Immunity to Trichinosis.* (19674)

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Several sources of radiation such as radium (1), X-rays(2) and radioactive cobalt(3) have been used to study the lethal action of ionizing radiation upon the isolated larvae of *Trichinella spiralis* and the encysted larvae in trichinous meat. Research along this line has been directed largely towards a possible application of radiation to destroy the larvae in infected pork. Semrad(2) gave 1,000 to 2,250 r (roentgens) in daily exposures of 250 r to rats infected with Trichinella larvae 1 day prior to the initial exposure to X-radiation. He reported a reduction in the number of larvae produced in irradiated rats. There have been no reports on the effect of radiation upon susceptibility and immunity to infection with *Trichinella spiralis*. It is the purpose of this paper to report an increase in susceptibility and the destruction of active immunity to Trichinella infection in mice following cobalt^{60} gamma irradiation.

Materials and methods. The gamma radiation source was composed of 21 pieces of cobalt^{60} arranged to give a uniform field at 12 inches. The intensity of the radiation at the present time is approximately 600 rep (roentgen equivalent physical) per hour and the LD_{50} for 28-day-old mice 750 (rep). All gamma radiation referred to in these experiments are whole-body exposures. Swiss mice from our own colony were used in the present study. These mice are free of Salmonella and internal metazoan parasites. The Trichinella larvae used for test infections were isolated by pepsin digest from stock mice with infections of 6 weeks duration. Known infective doses were determined by the dilution count method of McCoy(4) and concentrated in conical centrifuge tubes. Using a rubber bulb, the larvae were drawn with about 1 cc of tap water into a tapered, small bore pipette. While under ether anesthesia, the infective dose was administered directly into the stomach by passage of the pipette down the esophagus. All mice received test infections on a larvae per gram of body weight (lpg) basis. The infective doses were given within a 1- to 3-hour post-irradiation period.

Experimental and results. Several experiments were carried out to determine the effect of sublethal irradiation of the host upon susceptibility to Trichinella infection. The results of sublethal radiation upon 25 and 50 (lpg) infections in Exp. 1 and 2 are presented in Table I. Sublethal gamma exposure resulted in a significant increase in susceptibility of 4-week-old mice infected with 25 (lpg). With a 50 (lpg) infection, sublethal radiation increased the susceptibility to Trichinella infection by 63%. Exp. 3, Table I, shows the effect of administering 650 (rep) exposure to mice given a 50 (lpg) infection. This level of radiation increased the susceptibility to a 50 (lpg) infection by over 85%. These data clearly indicate that gamma radiation of 550 to 650 (rep) increased the susceptibility to Trichinella infections in mice.

A high degree of acquired immunity to a challenge infection of *T. spiralis* may be demonstrated in mice actively immunized with a sublethal active infection. Immunity to reinfection with Trichinella was first reported by Ducas(5). An experiment was designed to test the effect of 600 (rep) gamma radiation on the ability of Trichinella-immune mice to withstand a challenge dose. Sublethal active infections of 100 larvae (8 lpg) were given to 4-week-old mice as an immunizing infection. A description of the experimental groups and the per cent survival is summarized in Table II. The challenge dose of 1,500 larvae (75 lpg) was administered 3 weeks following the initial immunizing infection. In the group of

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* Research carried out at Brookhaven National Laboratory under the auspices of the Atomic Energy Commission.

TABLE I. Effects of Gamma Radiation from a Cobalt^{60} Source upon Susceptibility of Mice to *Trichinella spiralis* Infection.

Exp.	Group description	Gamma radiation rep.	No. mice	No. died	% died
I	Irradiated control	580	35	0	0
	" and infected	580	35	9	25.7
	Infected control	(No radiation)	35	0	0
II	Irradiated control	550	38	0	0
	" and infected	550	40	32	80
	Infected control	(No radiation)	42	7	16.6
III	Irradiated control	650	30	4	13.3
	" and infected	650	33	33	100
	Infected control	(No radiation)	32	0	0

Exp. I—Infective dose 300 Trichinella larvae (25 lpg). Exp. I and II—Infective dose 625 Trichinella larvae (50 lpg).

TABLE II. Effect of Gamma Radiation from a Cobalt^{60} Source upon Immunity to *Trichinella spiralis* Infection in Mice.

Group description	Gamma radiation rep.	No. mice	No. died	% survival
Irradiated control (no immunization)	600	40	1	97.5
Trichinella challenge infection (no immunization)	None	39	39	0
Trichinella challenge infection (immunized)*	"	34	9	74
Irradiated and Trichinella challenge infection (immunized)*	600	37	37	0

* Immunized with an active infection 100 *Trichinella spiralis* larvae; challenge infection 1500 larvae (75 lpg).

irradiated control animals, which received 600 (rep), 1 mouse died and 97.5% survived. All the mice died in the non-immunized Trichinella challenge group. The challenge dose thus constituted an LD$_{100}$. Seventy-four per cent of the non-irradiated, Trichinella-immune controls survived the challenging infection, while in the last group which were immunized, irradiated and challenged there were no survivors, showing that this amount of radiation is sufficient to destroy the active immunity produced by a previous infection with 100 *T. spiralis* larvae.

Acquired immunity to Trichinella infections has been reported to be localized in the intestines by Ducas(5) and McCoy(6). Culbertson and Kaplan(7) reported immunity to Trichinosis as a generalized response, the antibody being directed against the larvae which are developing into sexually mature adults in the intestine. There appeared to be no evidence of cellular reaction in the intestinal wall of immune rats(6). We have found that gamma radiation does not reduce the level of influenza virus neutralizing antibody present at the time of irradiation. We have also determined that cobalt^{60} radiation does not decrease either the amount of or the effectiveness of tetanus antitoxin in mice present at the time of irradiation(8). Precipitin tests of serum from irradiated Trichinella-immune mice and serum from non-irradiated immune mice exhibited an equal level in the ability to precipitate dilutions of precipitinogen prepared from Trichinella larvae, indicating that irradiation does not decrease the precipitins for Trichinella larvae which are present at the time of radiation. The effects of 680 (rep) gamma radiation upon total leukocytes per cu mm of peripheral blood are shown in Fig. 1. Each point on the curve represents the average total white cell count for 5 mice drawn at random from 2 groups of 80 mice. The sharp reduction in the total number of white cells in the circulating blood following gamma radiation represents a serious

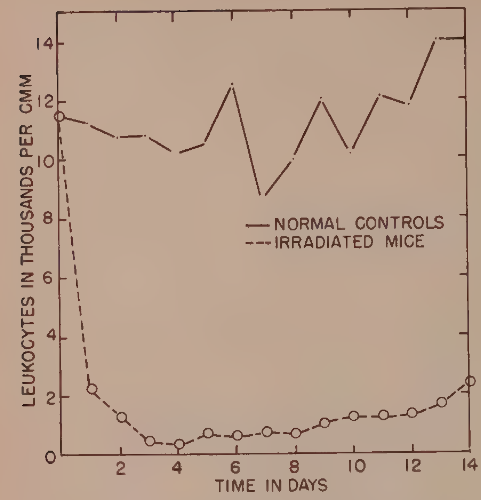

FIG. 1. Effect of gamma radiation 680 (rep.) upon level of leukocytes in peripheral blood of mice.

loss to the cellular defensive mechanism of an irradiated animal. It is indicated from these data that granular leukocytes and the lymphoid-macrophage system may be of greater importance in immunity to Trichinella infections than has formerly been considered.

Summary. 1. Cobalt^{60} gamma whole body irradiation of 550 to 650 (rep) increased the susceptibility of Swiss mice to infections with *Trichinella spiralis.* 2. Exposure of Trichinella-immune mice to 600 (rep) before administering a challenge infection destroyed their immunity to reinfection. 3. Data are presented on the effects of 680 (rep) radiation upon the total leukocyte count in the peripheral blood of mice. The sharp reduction in the number of circulating leukocytes may indicate that the role of cellular immunity should be emphasized more in acquired immunity to infections with *Trichinella spiralis.*

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Immunization of Rabbits with Type II Pneumococcal Polysaccharide.* (19675)

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The specific soluble substances of the pneumococci have been reported to be antigenic in man, mouse, horse, dog and cat(1), but all attempts to immunize rabbits with the type-specific polysaccharides have failed. For example, Avery and Morgan(2), Schiemann(3), and Avery and Goebel(4) reported attempts to immunize rabbits with pneumococcal polysaccharides, but they failed to detect type-specific precipitins, agglutinins, or protective antibodies in the sera of the treated rabbits. Schiemann(3) employed 1.0 to 1.5 mg of type II polysaccharide; Avery and Goebel(4) used as much as 18 mg and as little as 180 γ of type I pneumococcal polysaccharide in experiments to immunize rabbits. Although they failed to find type-specific antibodies, Avery and Goebel(4) were able to detect type I polysaccharide in the sera of their treated rabbits. It appeared likely that these investigators had employed "paralyzing" doses of the

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type-specific polysaccharides. This "immunological paralysis" with large doses of polysaccharide had been noted in mice(5). The purpose of this paper is to present evidence that small quantities of type II pneumococcal polysaccharide injected intravenously or intradermally are antigenic in rabbits.

Materials and methods. Type II pneumococcal polysaccharide, Lot 31T,‡ employed in this investigation contained 0.42% nitrogen and gave a positive test with Folin-Ciocalteau reagent(6,7). The viscosity of a 0.1% solution of the polysaccharide in 0.9% sodium chloride was 1.55 at 20.0°C. One-tenth gamma of the polysaccharide actively immunized mice against 10,000 LD of pneumococcus type II.

In one experiment each of 6 New Zealand white rabbits from 1700-2270 g in weight received intracutaneously on the right flank at weekly intervals 10 γ of pneumococcal type II polysaccharide in 1.0 ml of 0.85% NaCl. During the course of immunization each rabbit received 40 γ of type-specific polysaccharide.

In another experiment each of 6 New Zealand white rabbits weighing 1800-2500 g was inoculated intravenously with a saline solution containing 5 γ of pneumococcal type II polysaccharide per ml. Each rabbit received 14 injections of 1 ml amounts over a period of 4 weeks or a total of 70 γ of polysaccharide.

A sample of serum for control purposes was obtained from each rabbit before the start of the immunization schedules and a second sample was secured on the 7th or 8th day following the final injection of polysaccharide to test for specific antibodies. The sera from the animals in the same test group were pooled for storage in sealed ampules at —20°C.

The rabbits which had been bled for test sera were tested by the method of Goodner(8) for active immunity against pneumococcus type II. The test and control animals were challenged on the left flanks with 0.2 ml doses of varying dilutions of a 14-hour culture of pneumococcus type II grown in yeast-tryptose-phosphate broth containing 5% fresh rabbit blood. Rabbits failing to develop a fever, or edematous lesions and showing no loss of appetite or weight were considered to exhibit immunity(8).

Mouse-protection tests for assessing each pool of rabbit antiserum were performed by employing male or female Swiss mice, strain CFW. Each mouse received by intraperitoneal injection a mixture of 0.2 ml of serum and 0.3 ml saline several seconds before the administration by the same route of 0.5 ml of a diluted 15-16 hour culture of pneumococcus type I or II grown in blood broth. Groups of three 18-22 g mice were used for each dilution of bacterial suspension. The mice that survived for at least 96 hours were considered passively immune. The Quellung reaction, using type-specific antiserum and the peritoneal exudate of mice dead within 96 hours, was employed to determine the presence of pneumococcus type II.

Blood-broth cultures of pneumococcus type I or II employed to challenge the rabbits and mice were obtained by seeding blood-broth with the heart's blood of mice injected intraperitoneally with the fresh broth cultures of pneumococci. In the case of the 14-hour cultures of pneumococcus type II used to challenge the rabbits, 10-fold dilutions were made from equal volumes of yeast-tryptose-phosphate broth and saline. With the 15-16 hour cultures of pneumococci employed in the mouse-protection tests a 5-fold dilution was made first and then successive 10-fold dilutions.

Results. Table I shows that all 12 rabbits injected either intradermally or intravenously with pneumococcal type II polysaccharide exhibited signs of active immunity against an intradermal infection with type II pneumococcus. The test animals failed to develop fever and 5 had slight transitory lesions. All control rabbits challenged with dilutions of 10^{-1} and 10^{-2}, two at 10^{-3}, and two at 10^{-4} developed fever and edematous lesions.

Table II presents the findings of mouse-protection tests that employed the pooled serum from the 6 rabbits which had been immunized intradermally with type II pneumo-

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‡The authors are grateful to Dr. H. D. Piersma, Director of the Human Biological Section, Lederle and Co. for the generous supply of polysaccharide.

TABLE I. Active Immunization of Rabbits by Intradermal or Intravenous Injection of Type II Pneumococcal Polysaccharide

History of rabbits	Challenge with dilutions of type II pneumococcus* 10^{-1}	10^{-2}	10^{-3}	10^{-4}
Received intradermally type II polysaccharide	3/3†	3/3	——	——
Received intravenously type II polysaccharide	3/3	3/3	——	——
Normal controls	0/3	0/3	1/3	1/3

* The challenge dose was 0.2 ml of a diluted 14 hr blood broth culture given intradermally.
† Numerator = No. of rabbits exhibiting active immunity; denominator = No. of rabbits challenged.

TABLE II. Demonstration of Type-Specific Protective Antibodies for Mice in Serum of Rabbits Injected Intradermally with Pneumococcal Type II Polysaccharide.

Homologous challenge

Mice	Dilutions of homologous challenging organism† 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Injected with immune serum	0/3*	0/3	1/3	3/3	3/3	3/3	3/3	——	——
Injected with normal serum	0/3	0/3	0/3	0/3	0/3	0/3	0/3	——	——
Normal controls	——	——	0/3	0/3	0/3	0/3	0/3	0/3	2/3

Heterologous challenge

Mice	Dilutions of heterologous challenging organism‡ 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Injected with immune serum	0/3*	0/3	0/3	0/3	0/3	0/3	0/3	——
Injected with normal serum	0/3	0/3	0/3	0/3	0/3	1/3	1/3	——
Normal controls	——	0/3	0/3	0/3	0/3	1/3	1/3	3/3

* Numerator = No. of mice surviving at least 96 hr; denominator = No. of mice challenged.
† 0.5 ml of diluted 15 hr blood-broth culture of pneumococcus type II.
‡ 0.5 ml of diluted 15 hr blood-broth culture of pneumococcus type I.

TABLE III. Demonstration of Protective Antibodies for Mice in Serum of Rabbits Injected Intravenously with Pneumococcal Type II Polysaccharide.

Mice	Dilutions of homologous challenging organism† 10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}
Injected with immune serum	0/3*	0/3	0/3	3/3	3/3	3/3	3/3	——	——
Injected with normal serum	0/3	0/3	0/3	0/3	0/3	0/3	0/3	——	——
Normal controls	——	——	0/3	0/3	0/3	0/3	0/3	1/3	3/3

* Numerator = No. of mice surviving at least 96 hr; denominator = No. of mice challenged.
† 0.5 ml of diluted 16 hr blood-broth culture of pneumococcus type II.

coccal polysaccharide. It can be seen that 0.2 ml of the pooled immune serum protected mice against at least 10,000 LD of pneumococcus type II, but probably not against a single LD of pneumococcus type I. The pooled normal serum of the same 6 rabbits is seen to have been ineffectual in protecting mice against either pneumococcus type II or type I.

The data given in Table III show that the pooled serum of rabbits which had been immunized intravenously with type II pneumococcal polysaccharide protected the mice against almost 10,000 LD of type II pneumococcus, whereas the pooled normal serum of the same animals failed to protect against the homologous organism.

Neither of the 2 pools of immune serum gave a precipitin test with the type II pneumococcal polysaccharide, nor they did agglutinate a suspension of type II pneumococcus killed with heat and formalin.

Discussion. The results of these experiments afford evidence that the pneumococcal type II polysaccharide in minute amounts is antigenic in rabbits and suggest that other pneumococcal type-specific carbohydrates in extremely small quantities would also elicit the formation of mouse-protective substances in rabbits. In view of Felton's work(5) employing mice it is possible that the immune mechanism in rabbits in response to minute amounts of type-specific polysaccharide and perhaps even to heat-killed pneumococci could be "paralyzed" with large quantities of the same specific soluble substance.

Dubos(9) showed that heat-killed pneumococcus type I treated with bacteriolytic enzymes showed a loss of antigenicity in rabbits; these findings were based on the failure to detect type-specific precipitins in the sera of his experimental animals. It is possible that the sera of these rabbits contained mouse-protective antibodies.

Dubos and MacLeod(10) found that leucocytes contained an enzyme which inactivated the capsular antigen of pneumococcus type I *in vitro*. They suggested that this same enzymatic activity may take place in the skin of rabbits and thus account for the lack of type-specific antigenic response to the intradermal injection of heat-killed pneumococci. However, since the authors reported the failure of the enzyme to destroy the type-specific polysaccharide, one might predict from our findings that the intradermal injection of heat-killed pneumococci into rabbits would result in the production of type-specific mouse-protective antibodies.

Angevine(11) in studies on the antigenic response of rabbits to the intracutaneous injection of heat-killed pneumococcus type I reported the absence of type-specific agglutinins in the sera of the treated rabbits 33 days after the initial inoculation, but on continued intracutaneous injections, type-specific agglutinins were demonstrable 63 days after the first injection. If mouse protection tests had been performed, it is possible that mouse-protective substances would have been detected in the sera of the treated rabbits showing no agglutinin content after 33 days.

From our work one may postulate that the mouse-protective antibody elicited in rabbits by the type II pneumococcal polysaccharide is the type-specific anti-carbohydrate present in extremely small amounts. It was thought at first that this mouse-protective substance might be a univalent or so-called incomplete antibody. However, we were unable to show in a serum known to contain type II mouse-protective substances the presence of univalent antibodies employing the quantitative technic of Heidelberger *et al.*(12). Furthermore, precipitins were not demonstrable with the quantitative micro-technic described by Heidelberger and MacPherson(6,7). The failure to find in the mouse-protective serum univalent or precipitating antibodies employing quantitative technics is in accord with the findings of Stillman and Goodner(13). These authors suggested that the mouse-protective test was much more sensitive than the agglutination test. Of great importance, to an understanding of these results, Northrop and Goebel(14) showed that 0.5 γ of dissociated pneumococcal type I antibody N was capable of protecting mice against 1,000,000 LD of pneumococcus type I, whereas 16 γ were necessary to agglutinate heat-killed pneumococcal type I cells. Consequently an immune serum protective against 10,000 LD of pneumococcus type II would contain insufficient antipolysaccharide to be detected with the micro-technics employed by Heidelberger, *et al.* (6,7,11).

Summary. 1. Type II pneumococcal polysaccharide was found to be antigenic in rabbits. 2. Rabbits injected intradermally or intravenously with minute amounts of type II pneumococcal polysaccharide developed an active resistance to intradermal infection with type II pneumococcus. 3. The pooled serum of rabbits injected intradermally with the polysaccharide protected mice against 10,000 LD of pneumococcus type II, but not against pneumococcus type I. 4. The pooled serum of rabbits injected intravenously with the polysaccharide protected mice against almost 10,000 LD of pneumococcus type II. 5. It is suggested that previous failure to immunize rabbits with type-specific polysaccharides may be attributed to the use of excess polysaccharide resulting in "immunological paralysis." 6. The evidence shows that the mouse-protective antibody is the specific antipolysaccharide which occurs in amounts too small to be detected by quantitative precipitin technics. 7. An attempt to demonstrate univalency of the type-specific mouse-protective antibody failed.

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Development of Resistance to 4-Amino-N^{10}-Methyl Pteroylglutamic Acid (Amethopterin) by *Leuconostoc citrovorum*.[*] (19676)

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In children with acute leukemia, whose disease initially responds well to treatment with 4-amino-N^{10}-methyl pteroylglutamic acid (Amethopterin), there develops in the leukemic process, after 6 to 18 months, refractoriness to therapy(1-4). The elucidation of the mechanisms through which this resistance is established would be of extreme importance to the practical chemotherapy of this disease, and hence have been studied in several different systems. The induction of resistance to amethopterin in 2 strains of mouse leukemia has been demonstrated(5,6). The development has also been reported of an amethopterin-fast strain of *Streptococcus faecalis* which, in the absence of amethopterin, maintained its quantitative requirement of pteroylglutamic acid (PGA), but was capable of establishing maximum growth in media containing amethopterin, 4-amino PGA (aminopterin), and 4-amino pteroyl aspartic acid (amino-an-fol) in the absence of PGA(7,8). Studies with this resistant *S. faecalis*(9) have given some insight into the mechanism of the resistance, as well as the role of PGA and citrovorum factor (CF) in the process of cell metabolism.

Since CF has been shown to be a more direct competitor of aminopterin and amethopterin in *S. faecalis*(10), mouse leukemia (11), and human leukemia(12), it was thought again that a biological system specifically requiring CF might be of value for further study of amethopterin resistance.

Method. Leuconostoc citrovorum (ATCC No. 8081) was grown for 18 hours on the CF assay medium of Sauberlich and Baumann (13) in the presence of 1 mγ/ml of leucovorin (synthetic CF Lederle),[‡] the culture was centrifuged and washed once with saline and inoculated into the same medium with amounts of amethopterin ranging from 0-500 mγ/ml and incubated at 37°C. In 24 hours there was half maximum growth at 50 mγ/ml

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[†] Research Fellow of the National Cancer Institute.

[‡] Supplies of leucovorin were supplied through the kindness of Dr. James Ruegsegger, Lederle Laboratories Division, American Cyanamid Co.

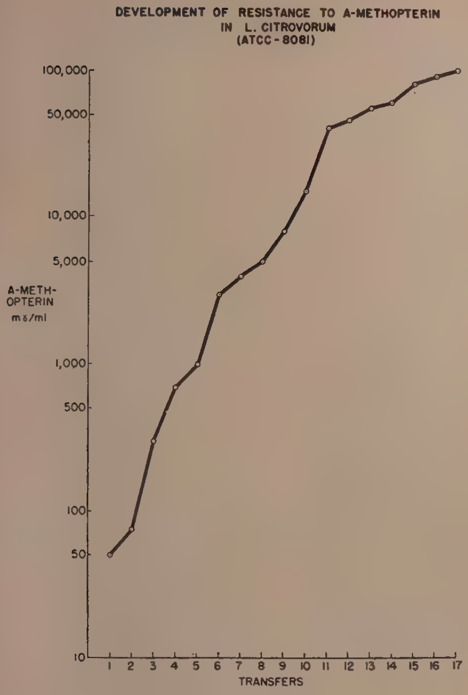

FIG. 1.

with maximum growth at the 0, 5, and 25 mγ/ml levels. After 7 days of incubation there was still no growth in the tubes containing 100 mγ/ml. After 6 days, tubes containing 25, 50, and 75 mγ/ml of amethopterin and 1 mγ/ml of leucovorin were inoculated from the 50 mγ tube and good growth was observed in 24 hours. This procedure was followed using one drop of the culture containing the highest concentration of amethopterin to inoculate 5 ml of fresh medium as soon as possible after growth appeared. Thus, through 17 transfers covering a period of 51 days a culture with a 2000-fold increase in resistance to amethopterin was selected. Comparative studies with the parent strain have been carried out both in the presence and absence of leucovorin.

Results. The gradual development of resistance as shown in Fig. 1 indicates a stepwise resistance pattern very similar to that of *S. faecalis*(7), even though slightly different methods were used in the induction of resistance. The growth rate of this amethopterin-fast organism, designated *L. citrovorum*/A, was approximately half that of the parent strain when high concentrations of amethopterin were used, *i.e.*, the growth obtained in 48 hours with the resistant culture was of the same magnitude as that of the parent strain in 18 hours. Therefore, it was arbitrarily decided to maintain the culture in a medium containing 20 γ/ml of amethopterin and 1 mγ/ml of leucovorin on which maximum growth was obtained in 18-24 hours. However, when small inocula were used, *i.e.*, one drop of a 1-100 dilution of a 20-hour culture, a 48-hour incubation period was found to give more reproducible results.

The requirement for CF (leucovorin) by the *L. citrovorum*/A has not changed from that of the parent sensitive strain, half maximum growth was obtained on 0.15 mγ/ml of leucovorin. Approximately the same amounts of PGA, 10-20 γ/ml, and N^{10}-formyl PGA, 50 γ/ml, were required for half maximum growth of each culture. When tested over a range of from 0.1 mγ to 1 mg per 2 ml of culture medium with prolonged incubation, and in the absence of leucovorin, none of the following compounds could be utilized for growth by *L. citrovorum*/A, amethopterin, aminopterin, amino-an-fol, 9-methyl PGA, 10-methyl PGA, 9,10-dimethyl PGA, or 4-amino-9, 10-dimethyl PGA.

A slight (10-fold) increase in resistance to 2,4-diamino-5(3′4′dichlorphenyl) 6-methyl pyrimidine (SK 5265), was noted (Table I) when both half maximum inhibition and complete inhibition levels were compared. This pyrimidine has been shown to have properties somewhat similar to amethopterin(14-17).

The difference between these 2 strains, therefore, seems to be manifest in the amethopterin resistance and a slight increase in resistance to SK 5265. Morphologically the 2 cultures appear to be identical and the inhibitory action of 5 antibiotics is also similar. Complete inhibition of each strain was obtained with 1.5 γ/ml of penicillin, 5 γ/ml of chloromycetin, 150 γ/ml of streptomycin; with aureomycin and terramycin, 500 γ/ml did not inhibit either strain.

Discussion. The development of resistance to amethopterin by *L. citrovorum* is somewhat

TABLE I. Inhibition of 2 Strains of *Leuconostoc citrovorum* by SK 5265.

CF ($m\gamma$/tube)*	$m\gamma$/tube of SK 5265	
	H.M.I. × 1000	Complete inhibition × 1000
	L. citrovorum 8081	
1	.5	3
100	5	20
	L. citrovorum/A	
1	5	30
100	50	100

* Total vol/tube was 2 ml.

comparable to that of *S. faecalis*(7). So far, however, it appears to be more related to that of penicillin(18), chloromycetin(19), aureomycin(18), and terramycin(20), since the resistance has not yet affected the physiology of the organism other than its ability to grow in the presence of extremely high concentrations of amethopterin. Unlike streptomycin resistance(21), the development of resistance to amethopterin took place over a long period of time and in a stepwise fashion. It differs from the amethopterin-fast *S. faecalis* in that *L. citrovorum*/A has not yet acquired the ability to utilize amethopterin, aminopterin, or amino-an-fol.

Summary. 1. A two thousand-fold increase in resistance to amethopterin has been induced in *L. citrovorum*. Since growth was slow at this high concentration of amethopterin, the culture was maintained on a medium with 400 times the concentration of amethopterin tolerated by the parent culture. 2. This amethopterin-fast variant has been maintained in the above medium for more than 5 months without any observed physiological or morphological changes.

We wish to express our appreciation to Miss Margaret Kennedy for technical assistance.

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Comparison of Antibacterial Activity of Single and Combined Sulfonamides.* (19677)

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Schweinburg and Rutenberg(1) reported that combinations of some of the sulfonamides were less bactericidal, in some instances, than the single agents which composed them. Although decreased antibacterial activity was most notable against gram negative organisms, it was also observed, to a lesser degree, with gram positive ones. Additive and even potentiating activity was produced by mixing some of the drugs. Differences in effect of the sulfonamide mixtures were related to variation in species and strain susceptibility. The purpose of the present report is to present confirmation of the work of Schweinburg and his coworker and to indicate the percentile incidence of the various types of reactions (additive, potentiating or depressing) which occur when sulfonamide drugs are combined.

Methods. All of the bacterial inocula were grown and the tests for inhibition by the sulfonamides carried out in peptone broth. Seventy-one strains of organisms were used: *E. coli* (7), *A. aerogenes* (5), *Salmonella* of various types (5), *Shigella* of different types (6), *B. subtilis* (3), alpha *Streptococcus* (8), beta-hemolytic *Streptococcus* (10), and *Staphylococcus aureus* (7).

The drugs studied were sulfadiazine (SD), sulfathiazole (ST), sulfamerazine (SM), and sulfamethazine (SMT). They were tested singly and in the following combinations: SMT+SD, SMT+ST, SD+ST, SD+SM, SMT+SD+ST, and SM+SD+ST. The technic of determining antibacterial activity was the same as that employed by Schweinburg and Rutenberg(1): "1000 mg % stock solutions of each sulfonamide in broth were prepared and brought to a pH of 8.2. Stock solutions of the sulfonamide combinations were prepared by mixing equal volumes of stock solutions of the individual sulfonamides. Serial dilutions of each of these stock solutions were prepared in concentrations of 750, 500, 333, 250, 100, 50, 25, and 10 mg % respectively. One cc of each of the 8 dilutions was placed in sterile Wassermann tubes. To each tube was added 0.1 cc of a bacterial suspension prepared by diluting a 24-hour culture so that 0.1 cc contained 500-5000 bacteria. A control tube containing broth without sulfonamide was also inoculated with 0.1 cc of the same bacterial suspension. All tubes were incubated at 37°C for 24 hours (or longer for streptococci)." The bactericidal titer was determined by making a subculture from each tube showing no growth onto a peptone-agar plate containing no drug; the amount of sulfonamide in the first clear tube yielding no growth on subculture was taken to indicate the bactericidal quantity of sulfonamide, either single or in combination.

Results. The reactions noted with each of the combinations of sulfonamides were classified as additive effect (A), potentiation (P), and decreased antibacterial activity (D). The following was considered to represent an additive effect: The bactericidal titer for a strain of *E. coli* was 100 mg % of sulfadiazine and 50 mg % of sulfathiazole. A mixture composed of equal parts of the 2 drugs inhibited growth of the organism at a level of 75 mg %. Also interpreted as an additive response was the following: A strain of *Salmonella* was killed by 100 mg % of sulfadiazine and by 100 mg % of sulfamerazine; a combination of the two, 50 mg % of each, showed a bactericidal level of 100 mg %. This type of reaction might be assumed to indicate an absence of any change in antibacterial activity. We prefer, however, to include it in the category of an additive effect because the mixture was bactericidal at the same level as each of the drugs when tested alone despite the fact that sub-effective quantities of each (50 mg %) made up the combination. A potentiative

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effect was defined as one in which a mixture of 2 or 3 of the sulfonamides was lethal at a concentration lower than that of the drugs used singly or that could be expected from mere addition of antibacterial activity. Thus, one strain of *Proteus vulgaris* was inhibited by 100 mg % of sulfadiazine and sulfathiazole, respectively. When the 2 agents were combined, the bactericidal titer was 25 mg %. The following was considered as demonstrating a decrease in antibacterial effect: A strain of *B. subtilis* was killed by 250 mg % of sulfamethazine, 100 mg % of sulfadiazine, and 50 mg % of sulfathiazole, respectively. When the 3 drugs were mixed in equal proportions, the organism grew well at a concentration of 750 mg % of the combination.

In Table I are presented the summarized data of the entire study in terms of the effect of combining several sulfonamides on their antibacterial activity. The results obtained using the drugs singly are not included in this report in order to conserve space. However, most of the strains of both the gram negative and positive organisms were inhibited by each of the agents approximately in the same range of concentrations as reported by Schweinburg and Rutenberg(1). The data in Table I are stated in relation to the activity of the single sulfonamides which composed the mixtures studied. For example, the number 3 under *E. coli* and SMT+SD in the column labeled D indicates that 3 out of 6 strains of this organism were less susceptible to a combination of sulfamethazine and sulfadiazine than they were to either used alone. Very striking was the marked variation in the reaction of different strains of a single organism to the same combination of drugs. Notable also were the differences in susceptibility of different species of bacteria to some mixtures of the drugs. These observations illustrate the unpredictability of the bactericidal potency of a sulfonamide combination. Certain of the mixtures depressed the antibacterial effect against most organisms. Thus, SD+SM and SD+SM+SMT were least active against the gram negative bacteria while these same combinations, in addition to several others, showed the greatest decrease in effectiveness when tested against gram positive cocci.

TABLE I. Effect of Combining Various Sulfonamides on Their Antibacterial Activity.

	Organism — No. of strains — Effect of sulfonamide mixture																													
Sulfonamide combination	*E. coli*(7)			*Ps. aeruginosa*(5)			*K. pneumonia*(6)			*Proteus vulgaris*(9)			*Aerobacter aerogenes*(5)			Salmonella(5)			Shigella(6)			*Bacillus subtilis*(3)			α-Strep.(8)			β-Strep. (10)			*Staph. aureus*(7)		
	P	A	D	P	A	D	P	A	D	P	A	D	P	A	D	P	A	D	P	A	D	P	A	D	P	A	D	P	A	D	P	A	D
SMT+SD	0	4	3	0	1	4	0	1	5	4	3	2	0	2	3	0	3	2	1	4	1	0	0	3	0	8	0	0	0	10	0	1	6
SMT+ST	3	3	1	0	4	1	4	1	1	4	5	0	0	4	1	3	1	1	4	1	1	0	1	2	0	0	8	0	2	8	0	4	3
SD+ST	1	4	2	0	3	2	3	1	2	2	7	0	1	2	2	0	2	3	0	4	2	0	3	0	0	0	8	0	2	8	0	5	2
SD+SM	0	2	5	0	0	5	1	5	0	6	3	0	0	0	5	0	0	5	0	2	4	0	0	3	0	0	8	1	8	1	0	4	3
SMT+SD+ST	0	5	2	1	0	4	4	1	1	0	7	2	0	0	5	1	3	1	0	4	2	0	1	2	0	0	8	0	9	1	0	1	6
SM+SD+ST	0	0	7	1	0	4	1	1	4	0	7	2	0	2	3	0	1	4	0	1	5	0	0	3	0	0	8	0	5	5	0	0	7

P = Potentiating effect; A = Additive effect; D = Decrease in bactericidal potency.

TABLE II. Effect of Various Combinations of Sulfonamides on Gram Negative and Positive Organisms.

Drug combinations	Organisms	Effect of drug combination, No., and % of strains					
		Additive effect		Potentiation		Decreased activity	
		No.	%	No.	%	No.	%
SMT+SD	Gram —negative,— 43 strains	18	41.7	5	11.6	20	46.7
SMT+ST		19	44.5	18	41.7	6	13.8
SD+ST		23	53.3	7	16.2	13	30.5
SD+SM		12	27.8	7	16.2	24	56
SMT+SD+ST		20	46.7	6	13.8	17	39.5
SM+SD+ST		12	27.9	2	4.6	29	67.5
SMT+SD	Gram —positive,— 28 strains	9	32.1	0	0	19	67.9
SMT+ST		7	25	0	0	21	75
SD+ST		10	35.7	0	0	18	64.3
SD+SM		12	42.8	1	3.5	15	53.7
SMT+SD+ST		11	39.2	0	0	17	60.8
SM+SD+ST		5	17.9	0	0	23	82.1

These data confirm the work of Schweinburg and his coworker for the gram negative bacteria. They indicate, however, that there may also be an even more marked reduction in bactericidal power for the gram positive cocci.

In Table II are presented the data, without relation to particular bacterial species, in terms of the percentage of strains of gram negative and positive organisms showing various types of reaction to the sulfonamide combinations studied. These results indicate that SD+ST was the combination producing an additive and SMT+ST a potentiative effect most frequently against the gram negative bacteria. The poorest mixtures were SD+SM and SM+SD+ST. It appeared, therefore, that the addition of sulfamerazine to any other single or pair of sulfonamides produced a marked depression of antibacterial activity. The presence of sulfathiazole, on the other hand, especially in the double combinations, increased the bactericidal potency.

With none of sulfonamide combinations was a remarkable degree of additive or potentiating effect observed when gram positive bacteria were used as the test organisms. SD+SM was the mixture which heightened the degree of bactericidal activity most often. Next in order of increased effect were SD+ST and SD+SMT. It is apparent, therefore, that for the gram positive organisms, the addition of sulfadiazine to a combination produced the greatest rise in antibacterial potency. Combining SMT+SD+ST resulted in the greatest reduction in growth-inhibiting effect against both gram positive and negative bacteria.

Summary and discussion. The data obtained in this study indicate that combining several of the sulfonamides in mixtures containing either 2 or 3 of these agents may produce 3 types of change in their antibacterial activity as compared to the effect of the drugs used alone; an additive, potentiating or depressing effect. The results point out clearly, as did the studies of Schweinburg and Rutenberg, that the effect of mixing sulfonamides on the bactericidal potency against gram negative and positive bacteria is almost totally unpredictable. This raises considerable question as to the usefulness of combined sulfonamide therapy in human infections. In order to be certain of the clinical value of any mixture, determination of the actual sensitivity of the infecting organism to it would have to be carried out. This makes effective application of sulfonamide combinations highly impractical.

In contrast to the observations of Schweinburg and his coworker, there was a high incidence, in the present study, of depression of antibacterial effect when sulfonamides were combined and the tests carried out with gram positive organisms. The discrepancy is probably related to the fact that different strains of bacteria were used in the 2 studies. This possibility is supported by the data indicating marked variation in the susceptibility of different strains of the same bacterial species to a single drug mixture. These studies suggest that the presence of sulfathiazole tends to increase and of sulfamerazine to decrease the lethal effect of combined sulfonamides on gram negative bacteria. On the other hand, the addition of sulfadiazine increases the bactericidal potency for gram positive ones. The combination producing the greatest depression of antibacterial activity for all organisms, regardless of their tinctorial reaction, appears to be one containing sulfamerazine, sulfadiazine, and sulfathiazole.

Conclusions. 1. The antibactericidal activity of various mixtures of sulfonamides is unpredictable. 2. The observed effect varies markedly with different species and with various strains of the same species of organism. 3. Depression of bactericidal potency against both gram negative and positive bacteria frequently results from combining sulfonamides. 4. The greatest and most constant decrease in antibacterial effect is produced by mixing sulfamerazine, sulfathiazole, and sulfadiazine. 5. These results suggest that combinations of sulfonamides may have little clinical usefulness in many instances, since they are often not as effective as the single agents which compose them.

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Studies on Passive Immunity in Poliomyelitis. V. Lansing Antibody Levels in Humans after Gamma Globulin Administration.* (19678)

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(Introduced by R. C. Parker.)

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Gamma globulin has been used successfully in man not only for the prevention or attenuation of measles, but also mumps(1) and infectious hepatitis(2,3). There is thus some justification for an investigation of gamma globulin in the prophylaxis of poliomyelitis; this possibility has been discussed by Hammon(4), who concludes that the available evidence warrants a large scale trial. The present paper reports a study that has been carried out to determine poliomyelitis antibody levels in serum after inoculation of the doses of gamma globulin likely to be used in such a study. Advantage has been taken of the fact that gamma globulin contains antibody to the Lansing as well as the other types of poliomyelitis virus(5-7), so that tests for antibody can be carried out in mice.

Materials and methods. Persons inoculated. It was necessary to examine the sera of a considerable number of persons in order to find those whose serum contained no Lansing antibody. Fifty-one children were bled at the Home of the Imperial Order of the Daughters of the Empire, Toronto. These children were from one to 11 years of age, and were hospitalized for tuberculosis, some being convalescent. The use of such patients offered the advantage that temperatures were regularly recorded, and any reactions would be quickly noted. Sera were tested for Lansing antibody by a screening test, the details of which are given below. The sera of 19 children were found to be free of demonstrable Lansing antibody. The sera of over 300 adults attending the Blood Bank at The Hospital for Sick Children, Toronto, were tested by a similar method, and the sera of 30 were found to be free of demonstrable Lansing antibody. It may be stated that in no case did any patient complain of more than mild local discomfort after the inoculation; there were no febrile reactions. *Gamma globulin.* Gamma globulin prepared from pools of human plasma was supplied through the courtesy

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of the American National Red Cross by E. R. Squibb & Sons, New Brunswick, N. J. The lot used has been studied in this laboratory, and contains sufficient Lansing antibody to protect mice against a cerebral challenge(7). *Inoculation procedures.* Six children were inoculated with gamma globulin by the intramuscular route in dosages of 5 to 15 ml, not more than 7.5 ml being given in one buttock. Blood samples were taken before the injection, 4 days thereafter, and at weekly intervals for 6 weeks. Fourteen weeks after the intramuscular injection, the children were again bled and were then given gamma globulin by mouth; 4 received the same dose as given intramuscularly, and 2 received double the previous dose. Blood was taken weekly for 6 weeks. Four adults were inoculated with 20 ml gamma globulin intramuscularly, 10 ml in each buttock. Blood was taken at 4 days and at weekly intervals for 6 weeks. At a time varying from 11-14 weeks after the intramuscular injection, 2 of the adults were given 20 ml of gamma globulin orally, and the other two 40 ml. Blood was taken for a period of 6 weeks. Full details are shown in Table I. *Tests for Lansing antibody.* Tests were carried out by the technic used in these laboratories for several years(8,9). In brief, to serial dilutions of serum was added sufficient Lansing virus-infected mouse pool to give a final amount of 100 LD_{50}. Virus-serum mixtures were held at room temperature for 1½ hours and then injected into groups of 10 or 20 mice. Mice were observed for 4 weeks. Appropriate virus controls were included in each batch of tests. In the preliminary screening tests, serum was used undiluted and a smaller dose of virus (35 LD_{50}) was added; mice were observed for 3 weeks.

Results. Tests on the sera of the persons inoculated with gamma globulin by the intramuscular route showed that only a small amount of neutralizing antibody was detected in the undiluted serum; no evidence of antibody was found in any test in which dilutions of serum were employed. The results of testing undiluted serum-virus mixtures are shown in Table I. In practically every instance, all of 20 mice died when inoculated with a mixture of virus and undiluted serum obtained

TABLE I. Lansing Antibody Content of Serum after Administration of Gamma Globulin.

	Intramusc. inoculation of gamma globulin									Interval between intramusc. and oral administration (wk)	Oral administration of gamma globulin								
		Mice surviving when inoculated with virus and serum obtained at following times*										Mice surviving when inoculated with virus and serum obtained at following times*							
				Week									Week						
Initials	Amt/lb body wt (ml)	Before inj.	Day 4	1	2	3	4	5	6		Amt/lb body wt (ml)	Before inj.	1	2	3	4	5	6
Children																		
LC	.24	0/20	4/19	13/20	4/20	13/20	7/20	8/20	5/20	14	.24	1/16	0/10	0/10	0/10	0/10	0/10	0/10
JMi	.42	0/20	1/20	6/20	5/20	0/20	1/20	0/19	0/19	14	.42	0/20	0/10	0/10	0/10	0/10	3/10	2/10
JMo	.13	0/20	5/19	5/20	1/19	0/20	0/20	3/20	0/20	14	.13	1/16	0/10	0/10	1/10	0/10	1/10	—
ER	.22	0/20	8/20	7/19	1/20	3/19	0/19	0/19	1/20	14	.22	0/19	1/10	0/10	1/10	2/10	0/10	2/10
PG	.25	0/20	7/20	3/20	7/20	3/20	6/20	4/19	5/20	14	.50	0/20	0/9	1/10	0/10	0/9	0/8	0/9
DR	.12	1/20	8/20	2/20	8/20	1/18	1/20	1/19	2/20	14	.24	1/20	0/10	0/10	0/9	0/10	0/10	0/10
Adults																		
DED	.11	0/19	4/20	4/20	2/20	2/19	0/20	0/20	1/20	11	.11	0/7	0/10	0/10	0/9	0/9	0/9	1/9
JDM	.14	0/20	1/20	0/19	1/19	0/20	0/20	0/19	0/20	14	.14	0/18	0/9	—	0/9	0/10	0/10	0/10
TBB	.14	2/19	7/20	1/19	3/20	2/20	1/17	4/19	5/20	13	.28	3/20	0/9	1/9	1/10	1/9	1/10	3/9
BM	.15	0/19	13/20	3/19	4/19	5/19	3/20	2/19	4/20	12	.30	0/19	0/10	0/10	0/10	0/10	0/10	0/10

* All tests carried out with a final amount of 100 LD_{50} of Lansing virus and undiluted serum.

before inoculation, indicating absence of antibody. However, when the samples taken at 4 days and one week after inoculation were similarly tested some mice survived. With certain persons some protective capacity was found in serum taken 2 weeks or more after inoculation. It will be noted that sera obtained after oral administration did not protect any mice.

Discussion. It can be concluded that after the inoculation of gamma globulin by the intramuscular route in doses varying from 0.1 to 0.4 ml per pound body weight only small amounts of antibody appear in the serum. Neutralizing antibody was present for 2 weeks or sometimes longer. It is probable that by titrating the sera in tissue culture, a technic which is sensitive to smaller amounts of antibody, a longer period of persistence could be demonstrated. After the oral administration of gamma globulin no serum antibody could be detected. The dosages of gamma globulin investigated are as high as can be given in field trials. Antibody levels, decline logarithmically, and the half life of I^{131} homologous gamma globulin in man is 12-14 days(10). Accordingly, the neutralizing effect conferred on the serum by a more potent antibody-containing preparation passively administered should persist for several weeks. In this connection, it is of interest to note that low levels of serum antibody are apparently quite adequate to protect cynomolgus monkeys against challenge by the oral route(11). It is possible that quite small amounts of antibody may likewise serve to protect humans against virus entry through the pharynx or gastrointestinal tract.

Summary. 1. The sera of 51 children and over 300 adults were first tested for the presence of Lansing virus neutralizing antibody. 2. Of those whose serum contained no demonstrable antibody, 6 children and 4 adults were given gamma globulin intramuscularly in doses from 0.1 to 0.4 ml per pound body weight. 3. Blood was taken at frequent intervals for 6 weeks and tested for antibody. A small quantity of antibody was present in the serum, as shown by a protective effect of the undiluted sample in mice. This antibody was detected in some cases for a few weeks. 4. No antibody was found in the serum of the same 10 persons following the oral administration of gamma globulin.

We are grateful to Dr. Gladys Boyd and to the medical and nursing staff of the Home of the Imperial Order of the Daughters of the Empire, Toronto, for their kind co-operation in this study.

We also acknowledge the facilities provided by Dr. W. L. Donohue at the Blood Bank of The Hospital for Sick Children, Toronto, and for much practical assistance by Miss Isabel Scott and Miss Dorothy Lamontaigne.

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Role of Externally Produced Hydrogen Peroxide in Damage to *Paramecium aurelia* by X Rays.* (19679)

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It has been suggested repeatedly that the effects of X-rays on cells are due, at least in part, to hydrogen peroxide produced by the irradiation of water. Not all investigators agree that it plays a prominent role, but it seems clear that this substance must be considered carefully in any investigation of radiation damage. We have been able to show that it is of major importance in the production of certain kinds of nongenetic effects in *Paramecium aurelia* but only under certain circumstances. We have not been able to demonstrate any effect upon the genetic mechanism.

Methods. A General Electric Maxitron 250 machine was used at 250 kvp and 30 ma. The machine had an inherent filtration of 1 mm of Al and no filtration was added. Measurements of intensity were made in air with a nylon Victoreen thimble chamber. This method cannot be considered to give more than a rough approximation to the intensity because of the large soft component. Intensities measured in this way were 70 and 15 kr/minute at the 2 positions used. The animals were irradiated in thin liquid preparations by methods to be discussed elsewhere(1). Stock 90 of variety 1 of *P. aurelia* was used throughout and all animals were selected to be in the first half of the interval between successive divisions.

After exposure, the irradiated animals, usually 30 for any one treatment group, were isolated individually in depression slides. The next day the number of animals in each depression was recorded. From these records, the percentage of survival and the average number of divisions among the survivors were calculated. These 2 quantities are considered to be measures of nongenetic effects for reasons discussed by Kimball and Gaither(2). Genetic effects were investigated by allowing the descendants of some of the irradiated animals, usually 20 from each treatment group, to undergo autogamy and isolating 25 autogamous animals from the descendants of each irradiated animal. At low doses, the percentage of autogamous animals that produced a large population in 4 days was used as a measure of genetic effect; whereas, at high doses, the percentage surviving at 4 days was a more convenient measure. The procedure for low doses was described by Kimball(3).

The culture medium (C) used in all these experiments was a lettuce infusion inoculated with *Aerobacter aerogenes* one day before use. In a number of investigations, irradiation and other treatments were carried out in a medium (D), consisting of 3 parts of boiled C to 97 parts of distilled water or 0.001 M KH_2PO_4 adjusted to pH 7. Just before treatment, the paramecia were transferred through 6 changes of this medium by capillary pipette, and groups of about 50 washed animals were transferred into separate depressions of D. The dilution of the original culture medium was at least 1:50,000 and usually greater. All exposures to H_2O_2 were made in this medium.

Effect of D medium and its various modifications. A comparison between animals irradiated in C and in D medium is given in Fig. 1. In each experiment, a group of paramecia in C medium and one in D medium were exposed simultaneously to the same dose. In part of the experiments, more than one dose was given. Points in Fig. 1 are averages for similar doses over all experiments (an average of 3 per point). Considerable variation was found from experiment to experiment in the response to a given dose, but consistent dose curves were obtained within experiments in which more than one dose was given. It is clear from the figure that there was a greater nongenetic effect in animals irradiated in D than in C medium. However, there was no appreciable influence of the medium upon

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* The work was performed under Contract No. W-7405-Eng-26 for the Atomic Energy Commission.

† The authors are indebted to Misses Mary Kathryn King and Catherine Stacy for their assistance in this work.

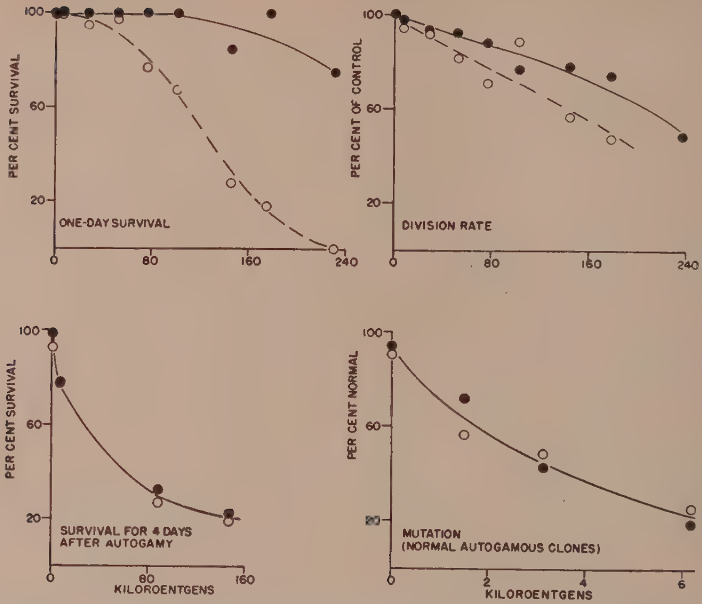

FIG. 1. Effect of culture medium upon action of X rays measured by % survival for one day, divisions per day as a % of the control, % survival for 4 days after autogamy, and % normal clones 4 days after autogamy. Part of the irradiations were at 70 kr/min and part at 15.5 kr/min. ○ = D medium. ● = C medium.

genetic effects either at high or low doses.

The protective action of C was destroyed either by filtration through an ultrafine sintered glass filter or by boiling with no filtration. Catalase (either a solution of crystalline enzyme or a partially purified commercial preparation) added to D medium before irradiation afforded nearly the same protection as C medium. Boiled catalase was without effect as was also crystalline bovine albumin. These results suggest that the protective action of C medium may be due to the catalase content of the bacteria which it contains. Organic ingredients in the lettuce infusion and in the boiled, "bacterized" medium were certainly much less effective than catalase. The hypothesis that the protection is due to the catalase content of the bacteria is supported by the fact that H_2O_2 added to C medium is fairly rapidly destroyed, while little, if any, destruction was brought about by boiled C medium.

Effect of irradiated D medium and of hydrogen peroxide. The results of the preceding section suggest that a major part of the non-genetic damage to *Paramecium* irradiated in D medium is due to H_2O_2. To test this conclusion, exposures were made to H_2O_2 in D medium and to irradiated D medium. All irradiations were made at 15.5 kr/minute. Just before each experiment, the concentration of the analytical grade "30%" peroxide was determined by titration with $KMnO_4$. A series of dilutions were used as standards for the determination of the amount of H_2O_2 in the irradiated medium (D), using the colorimetric method of Savage(4) which measures the iodine liberated from KI. Fig. 2 shows the results of the tests for peroxide at several different doses of X-rays.

Paramecia were exposed to the various concentrations of H_2O_2; and, at the same time, others were exposed to the irradiated fluid. Both had a marked effect on survival and division rate as shown in Fig. 3. While much variability was found, there seems little question that this substance can account for a considerable portion of but not all the effect of irradiated fluid. It can be estimated from Fig. 3 that the concentration of H_2O_2 needed

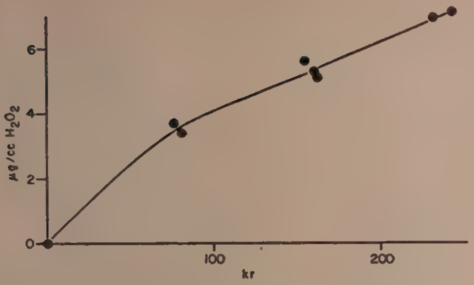

FIG. 2. Concentration of H_2O_2 produced by various doses of X rays to 20 cc of D medium in 10 cm petri dishes. Unfiltered beam. Measured intensity, 15.5 kr/min.

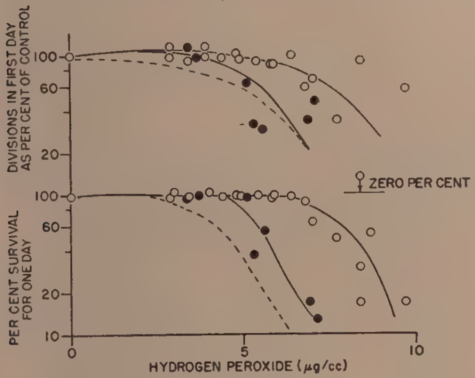

FIG. 3. Divisions in the first day as % of the control and % survival for one day plotted against the concentration of H_2O_2. The data for peroxide and irradiated fluid were obtained from 3 experiments in which the 2 were used simultaneously. The peroxide concentration for the irradiated fluid was obtained by colorimetric comparison with the peroxide solutions, using the KI-starch method The curve for direct irradiation is taken from Fig. 1. The dose in kr was converted to μg/cc of H_2O_2 using the curve in Fig. 2. ○ = H_2O_2 in D. ● = Irradiated D. – – – = Direct irradiation in D.

to produce the same effect as the irradiated fluid was about 1.3-1.5 times that found in the latter. In other words, two-thirds to three-quarters of the effect could be accounted for by the H_2O_2 which was formed. It is of interest in this connection that irradiation but not H_2O_2 destroyed the faint yellow color of the D medium. The change was not detectable at the wave length (660 mμ) used for the colorimetric determinations and so could not have produced a serious discrepancy in the readings. However this observation, together with the results in Fig. 3, shows that X-irradiation produces changes in the medium which cannot be accounted for by the H_2O_2 which it produces.

A comparison between direct irradiation and exposure to irradiated medium is also made in Fig. 3. The curves from Fig. 1 were replotted after converting kr to μg/cc of H_2O_2 using Fig. 2. The comparison is subject to errors of at least 3 kinds. The data in Fig. 1 were obtained by exposing the animals in small drops; whereas, the fluid was irradiated by exposing 20 cc in a Petri dish of 10 cm diameter covered with a thin piece of mica. Thus the actual dose to the animals was probably greater than that to the fluid. The time of exposure to irradiated fluid was variable in the case of directly irradiated paramecia and was probably greater on the average than the 10 minutes used in the experiments in Fig. 3. Both these factors would make the effect of a given dose measured in air greater upon directly irradiated animals. However, directly irradiated animals were exposed to gradually increasing concentrations of the active substances; whereas, those exposed to irradiated fluid were put immediately into the final concentration. Thus, for equal exposure times, the latter would be more effective. Considering these opposing sources of uncertainty in the comparison, it can be concluded that there is no convincing evidence that directly irradiated animals are affected to a greater extent than would be expected from the action of irradiated medium alone. However, direct absorption of energy within the paramecia is not excluded as a small contributing factor.

Further evidence that H_2O_2 was, in considerable part, responsible for the activity of irradiated medium and peroxide-treated medium was furnished by the demonstration that the addition of catalase destroyed the detectable activity in both cases. However, the possibility remains that organic peroxides formed from H_2O_2 and destroyed by catalase were involved.

Neither the irradiated medium nor H_2O_2 produced any detectable genetic effect as measured by the percentage of normal clones after autogamy even at doses at which only about 10% of the animals survived the first day. This is in good agreement with the fact, as shown in Fig. 1, that the medium in which irradiation is carried out is without appreciable influence on genetic effects. Thus the marked genetic effects produced by the high doses used must have been due entirely to energy absorbed directly in the cell.

Discussion. A number of investigators have demonstrated that substances produced in the medium are responsible for part of the effects of X-rays. Some of these investigators(5) have implicated H_2O_2, while others(6) have suggested other substances, stable or unstable. In particular, Barron *et al.*(6) have presented rather strong evidence that H_2O_2 could not be of much importance in the inhibition of the respiration of sea urchin sperm by irradiated sea water. The work of Wyss *et al.*(7), and Dickey *et al.*(8) suggests that H_2O_2 may not, in itself, be a mutagen for microorganisms, but that organic peroxides formed by its reaction with organic compounds are mutagenic. However, Wagner *et al.*(9), and Demerec *et al.*(10) have obtained evidence which suggests that H_2O_2 itself may be a mutagen. In addition Thoday and Read(11), and Giles and Riley(12) have suggested that H_2O_2, produced locally in the cell, may be responsible for chromosome breakage by ionizing radiations.

The data in the present paper are most easily interpreted on the hypothesis that H_2O_2 and at least one other fairly stable injurious substance are formed on irradiation of D medium and are responsible for most of the effect upon paramecia directly irradiated in this medium. It is possible that the H_2O_2 formed by irradiation reacts with organic ingredients in the medium to form organic peroxides which, in turn, produce the effect. In this case, we would have to assume, with Wyss *et al.*(8), that catalase destroys organic peroxides. Even granting that this is so, it should not invalidate the comparison between irradiated medium and medium to which peroxide was added, since organic peroxides should be formed in the same amounts in the latter. The difference in effectiveness which was found can be attributed only to some action of the X-rays which does not involve H_2O_2 as an intermediate step. One form that this action could take is the production of another injurious substance. However, it is also possible that the medium was altered in such a way that it protected the paramecia against the action of H_2O_2, not by the destruction of the peroxide, but by altering the paramecia to make them more resistant to it.

The failure to find a postautogamous effect of medium with H_2O_2 added and of irradiated medium could be interpreted to mean that this substance was not mutagenic. However, in the light of the evidence on other organisms cited in a previous paragraph, it seems more probable that H_2O_2 was unable to penetrate to the micronuclei in sufficient concentration to produce an effect. *Paramecium* is known to contain catalase (Holter and Doyle)(13), and the micronuclei are usually situated some microns below the surface. Thus it seems quite possible that the H_2O_2 or organic peroxides would have more difficulty in penetrating to them than to the nuclei of the microorganisms upon which positive tests have been obtained. The corollary to this hypothesis is that the materials whose alteration leads to death in one day and also to division delay are located in the surface layers of the *Paramecium* cell. In any case, it is clear that the high rate of mutation (80% or more of exautogamous clones not normal) induced by direct irradiation with doses of the order used in these studies could not be due, except to a negligible extent, to substances produced in the medium.

In assessing the importance of H_2O_2 produced outside the cell in the genesis of radiation damage within it, one must bear in mind that radiation damage to cells can, potentially, be brought about by a variety of mechanisms. The production of H_2O_2 is only one of these, and its relative importance is probably quite different under different circumstances of irradiation and with different effects of the radiation. It is not surprising, then, that estimates of its importance have varied so widely.

Summary. The production of nongenetic death and division delay by X-rays is greatly enhanced if the paramecia are irradiated in a dilute medium with very few bacteria rather than the usual lettuce infusion with many bacteria. This effect is shown to be accounted for almost entirely by relatively stable sub-

stances produced by irradiation of this medium. About two-thirds of the total effect can be attributed to H_2O_2 or its reaction products, while the rest results from an action on the medium not due to H_2O_2. On the other hand, the frequency of genetic changes is not affected by the medium in which the organisms are irradiated nor does irradiated medium or medium to which H_2O_2 was added induce such changes. The failure to find an effect upon the genetic mechanism is interpreted as probably the result of the failure of the substances produced in the medium to reach the micronuclei which are situated some microns below the cell surface. The corollary to this is that the nongenetic changes are due to alterations in the superficial layers of the cell.

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Spectrophotometric Studies on Mixtures of Purified Diphtherial Toxin and Ferriprotoporphyrin IX.*† (19680)

IRWIN H. LEPOW. (Introduced by Louis Pillemer.)

From the Institute of Pathology, Western Reserve University, Cleveland, O.

Pappenheimer(1-3) has proposed that diphtherial toxin is the protein moiety of diphtherial cytochrome b. Direct evidence for this concept would be available if it could be demonstrated that purified diphtherial toxin interacts with iron-porphyrins in a limiting molar ratio of 1:4 to form complexes spectrophotometrically and enzymatically related to cytochrome b. In other porphyrin-protein systems, specific interactions occur *in vitro* under a variety of experimental conditions. In the case of hemoglobin synthesis, the heme-globin linkage is not affected by considerable alteration of the protohemin‡ structure(4). Thus, mesohemoglobin and hematoglobin have been prepared by Hill and Holden(5) and synthetic hemoglobins from rhodohemin, diacetyldeuterohemin and pheohemin-b by Warburg and Negelein(6). All of these compounds combined reversibly with oxygen. Experiments on the recombination of the protein moiety of horseradish peroxidase with various hemins have been summarized by Maehly(7). The formation of methemalbumin has been shown by Rosenfeld and Surgenor(8) to occur over a wide pH range and to be unaffected by small changes in ionic

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† It is a pleasure to acknowledge the constant interest and encouragement of Doctor Louis Pillemer and the very able technical assistance of Miss Judith Bentoff.

‡ The nomenclature and terminology of Lemberg and Legge(4) is used in this paper when designating porphyrin derivatives and complexes.

strength and the specific nature of the buffer ions.§ Serum albumin also binds bilirubin and protoporphyrin(9).

Rawlinson and Hale(10,11) have shown that the major iron-porphyrin present in *cells* of *C. diphtheriae* is ferriprotoporphyrin IX, which is also considered to be the prosthetic group of mammalian cytochrome b(4). The possibility exists, therefore, that diphtherial toxin would interact with hemin (ferriprotoporphyrin IX chloride) if, indeed, diphtherial toxin were the protein moiety of diphtherial cytochrome b. Accordingly, the present report concerns results of spectrophotometric examinations of purified diphtherial toxin-hemin mixtures and the failure to obtain evidence for the formation of specific complexes.

Experimental. Materials. The highly purified *diphtherial toxin,* previously described (12), contained between 2250 and 2420 flocculating units/mg protein nitrogen. The preparations were at least 85% electrophoretically homogeneous in pH 7.5-7.6 phosphate buffer of ionic strength 0.2. The protein molarity was calculated assuming a mean molecular weight of 72,000(13) and a nitrogen-to-protein factor of 6.25. *Human serum albumin, Fraction V,* was prepared according to the method of Cohn and his associates(14), and was at least 95% electrophoretically homogeneous in pH 8.6 barbiturate buffer of ionic strength 0.1. The molarity of solutions was calculated, using a molecular weight of 69,000 and a nitrogen-to-protein factor of 6.25. *Crystallized hemin* (ferriprotoporphyrin IX chloride, Eastman Kodak) was recrystallized 3 times from pyridine-chloroform by the method of Fischer(15). Solutions were prepared by dissolving an accurately weighed sample of the 3 times recrystallized hemin in 10 ml of 0.1 N NaOH and immediately diluting to 1 liter with distilled water. The solutions were used within a 4-hour period and then discarded(8). *Spectrophotometric measurements* were made in the Beckman model DU spectrophotometer with Corex cells of 1 cm optical path.

Effect of molar ratio of hemin to toxin. Molar ratios of hemin to toxin of 0.5:1, 1:1, 2:1, 4:1, and 8:1 were prepared by adding calculated volumes of hemin solution, previously adjusted to ionic strength 0.15 with sodium chloride, to aliquots of an 8.1 x 10^{-6} M toxin solution in pH 7.4 phosphate buffer of ionic strength 0.15. The absorption spectra were measured at 380-640 mμ after initial mixing, after incubation for one-half hour at 38°C, after further incubation for one hour at 38°C, and after standing overnight at 0°C. The spectra were compared with those of freshly prepared hemin solutions in the absence of toxin and of a toxin solution in the absence of hemin. All samples were read against a blank consisting of the appropriate proportions of phosphate buffer and 0.15 M sodium chloride.

No evidence for the interaction of diphtherial toxin with hemin was observed in the 16-fold range of molar ratios tested. Reversal of the order of mixing of the reagents was without effect. Four to one molar ratios of hemin to diphtherial toxin at pH 7.4 and ionic strength 0.15 flocculated with antitoxin to the same titer as toxin controls. The Minimum Reacting Dose in the rabbit was also unaffected by the presence of hemin.

Effect of pH and buffer ions. Glycine, veronal, acetate and phosphate buffers were prepared in overlapping pH values from pH 2-12 at ionic strength 0.15. One volume of hemin solution, adjusted to ionic strength 0.15 with sodium chloride, was added to one volume of an 8.1 x 10^{-6} M solution of diphtherial toxin in 0.15 M sodium chloride. Two volumes of buffer were added and the solutions incubated at 38°C for one-half hour. The pH was then measured and the samples read in the spectrophotometer at 380-640 mμ. The final ionic strength of each sample was 0.15. The molar ratio of hemin to toxin was constant at 2:1. The spectra of hemin solutions in the absence of toxin under the same conditions of pH, ionic strength, incubation time, and temperature were also obtained.

The results at 3 typical pH values are shown in Fig. I. An increase in extinction in

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§ The author wishes to thank Drs. Rosenfeld and Surgenor for making available a manuscript of their paper prior to its publication. The technics employed in the present study were patterned after those of Rosenfeld and Surgenor(8).

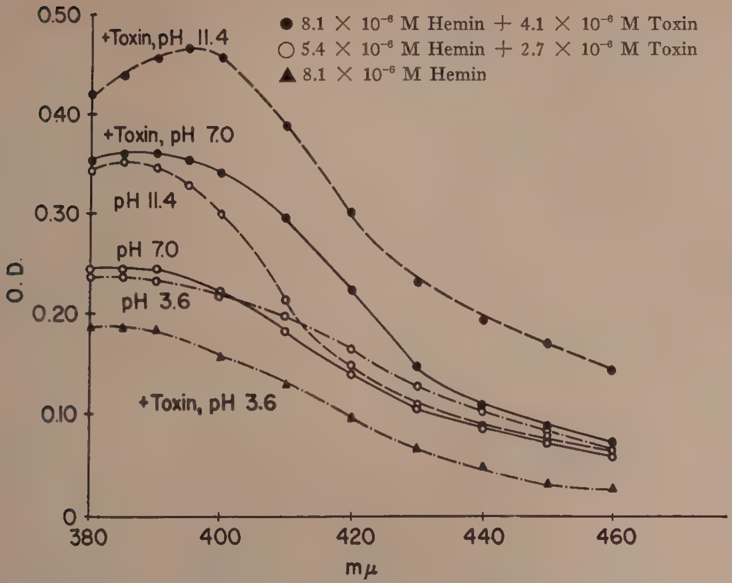

FIG. 1. Comparison of absorption spectra of hemin in presence and absence of diphtherial toxin at representative pH values in buffers of ionic strength .15: pH 3.6 veronal, pH 7 phosphate, and pH 11.4 glycine-sodium hydroxide.

the presence of diphtherial toxin was observed at all pH values where the toxin remained soluble. This non-specific dispersing effect of proteins on hemin solutions has been observed with other protein-hemin systems(8). A shift in the hemin absorption maximum in the presence of diphtherial toxin was noted only at pH values exceeding pH 10.

Precipitation occurred at pH 4.4, 4.9 and 5.6 when acetate or veronal buffers were added to toxin or to toxin-hemin mixtures. The precipitates were insoluble in water and in pH 2-10 buffers of ionic strengths 0.15 but soluble in dilute sodium hydroxide. The latter solutions, when prepared from toxin-hemin mixtures, gave absorption spectra of the type shown for pH 11.4 in Fig. 1. No spectrophotometric evidence for specific interaction could be obtained when toxin-hemin mixtures precipitated at pH 5.0 with acetate buffer were shaken at 0°C in a 10 KC sonic oscillator (Raytheon, 150 watts) for 10, 20, 30 or 60 minutes.

Effect of ionic strength. One volume of hemin solution in 0.001 N NaOH was added to one volume of a 6.16 x 10^{-6} M aqueous solution of diphtherial toxin to give a molar ratio of hemin to toxin of 4:1. Two volumes of buffer of appropriate concentration to give the desired final ionic strength were then added. The absorption spectra from 380-600 mμ were compared with hemin blanks consisting of the above system except for the substitution of water for toxin. Samples were prepared and measured in this manner at ionic strengths 0.05, 0.10, and 0.32, each at pH 2.0 (glycine buffer), pH 4.0 (acetate buffer), pH 7.1 (phosphate buffer), and pH 9.4 (glycine buffer).

No evidence for the specific interaction of diphtherial toxin was observed under any of the above conditions. The absorption spectra varied with pH as previously described but were essentially independent of ionic strength. The non-specific increase in extinction in the presence of toxin was observed at all wavelengths and at all pH values and ionic strengths.

Comparison of diphtherial toxin-hemin and serum albumin-hemin mixtures under alkaline conditions. The shift in absorption spectra observed when hemin was added to diphtherial toxin above pH 10 prompted a comparison of the spectra of toxin-hemin and albumin-hemin mixtures under the same conditions of alkalinity in the presence and absence of a reducing agent. Qualitative examination of 2:1 molar ratios of hemin:protein in dilute sodium hydroxide showed identical absorption maxima for diphtherial toxin and serum albumin hemichromes at 392-398 mμ and a very diffuse maximum at 560-580 mμ. Reduction with sodium hydrosulfite gave peaks at 423-424 mμ, 527-528 mμ, and 557-558 mμ for both hemochromes. These results were highly indicative of a non-specific reaction between hemin and denatured protein.

A stoichiometric study was carried out by adding one volume of varying concentrations of hemin to one volume of a constant concentration of diphtherial toxin or human serum albumin. The mixture was brought to 1% Na_2CO_3 by the addition of an equal volume of 2% Na_2CO_3. Hemin samples were prepared in the same manner, substituting water for the toxin. The blank consisted of 1% Na_2CO_3. A small amount of hydrosulfite was added to each sample and the optical densities measured at intervals of 10 minutes at 424, 528, and 558 mμ until the extinction began to decrease. The maximum reading at each wavelength was used for comparison of extinctions at the various molar ratios tested. Measurements on the hemichromes were made at 395 mμ immediately after mixing the reagents and prior to the addition of hydrosulfite.

The results were qualitatively identical at each of the 3 hemochrome absorption maxima. The optical density of the reduced albumin-hemin mixtures was found to be a linear function of the molar ratio between 0.5:1 and 8:1. The reduced toxin-hemin curve was superimposable on the albumin-hemin curve up to a molar ratio of about 4:1. Beyond this point, a small but reproducible decrease of slope was observed. Neutralization of 4:1 hemin-toxin alkali hemochromes resulted in dissociation. A typical experiment at the 424 mμ hemochrome absorption maximum is shown in Fig. 2. The optical densities of the albumin-hemin mixtures have been corrected to the same concentration as the toxin-hemin mixtures and the curves for each protein are directly comparable.

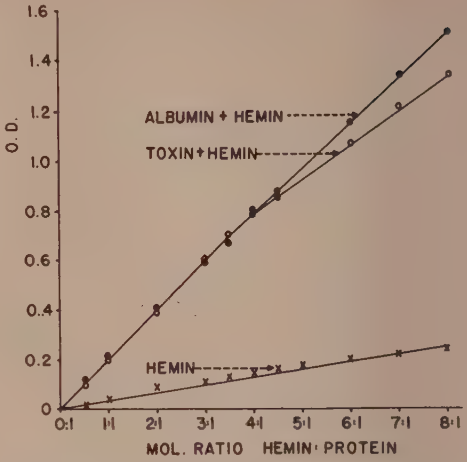

FIG. 2. Comparison of human serum albumin and diphtherial toxin alkaline hemochromes at 424 mμ.

The albumin and toxin hemichrome absorptions at 395 mμ were identical linear functions of the molar ratio of hemin to protein. The slight break in the toxin hemochrome curve at 4:1 was not observed with the oxidized mixtures.

Discussion. Diphtherial toxin does not specifically interact with hemin under the conditions investigated. Present knowledge of protein-porphyrin interactions would lead to the prediction that such an interaction would occur *in vitro* if diphtherial toxin were the protein moiety of cytochrome b. However, it cannot be assumed that the diphtherial toxin-hemin system necessarily falls within the generalizations previously cited for other protein-porphyrin interactions. The results of this study may only be interpreted as a failure to provide evidence for the Pappenheimer proposals on the primary relationship between diphtherial toxin and cytochrome b.

Summary. Diphtherial toxin-hemin mixtures prepared under a wide range of conditions of pH, ionic strength, buffer ions, molar ratio of hemin to toxin and incubation time have been examined spectrophotometrically. Combination of purified diphtherial toxin with hemin was observed only under conditions leading to non-specific alkali hemi- and hemochrome formation. The significance of these findings to the Pappenheimer proposals relating diphtherial toxin to cytochrome b is discussed.

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Effect of Certain Plant Growth Substances on Oxidative Phosphorylation In Rat Liver Mitochondria.* (19681)

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Recent work has resulted in technics whereby mitochondria from a number of mammalian tissues can be isolated by differential centrifugation and their metabolism studied using conventional procedures(1). It appears that the mitochondria are the chief intracellular sites of oxidative phosphorylation(2). A number of compounds, among them dinitrophenol(3,4) and the barbiturates (5), have been shown to uncouple phosphorylation from oxidative processes with a concomitant increase in respiration. Bonner(6) reported that 2,4-dinitrophenol in concentrations which inhibited section growth was a powerful promoter of *Avena* respiration. The effects of plant growth substances on certain plants is reflected in increased total respiration, with or without accelerated growth and development(7). It was suggested(8) that perhaps 2,4-dichlorophenoxyacetic acid (2,4-D) and other plant growth substances might be uncoupling agents. The above considerations led us to investigate the effects of various plant growth substances on a mammalian tissue system which would test this possibility.

Experimental. General procedures and methods were similar to those previously described(5,9). Rat liver mitochondria were isolated according to the methods of Schneider and Hogeboom(10). In the experiments designed to demonstrate a stimulatory effect on respiration the phosphate-deficient system of Judah(3) was used. The mitochondria used in these experiments were washed 3 times with 0.25 M sucrose in order to remove most of the inorganic phosphate. Other experimental details are given in the figure legends.

Results. In the experiments designed to show the effect of 2,4-D on oxidative phosphorylation (Fig. 1) it was found that this compound was a potent uncoupling agent. At concentrations of 2,4-D as high as 1 x 10^{-3} M there was little effect on respiration in the test system and yet the P:O ratio fell to 20%

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† Fellow of the American Foundation for Pharmaceutical Education.

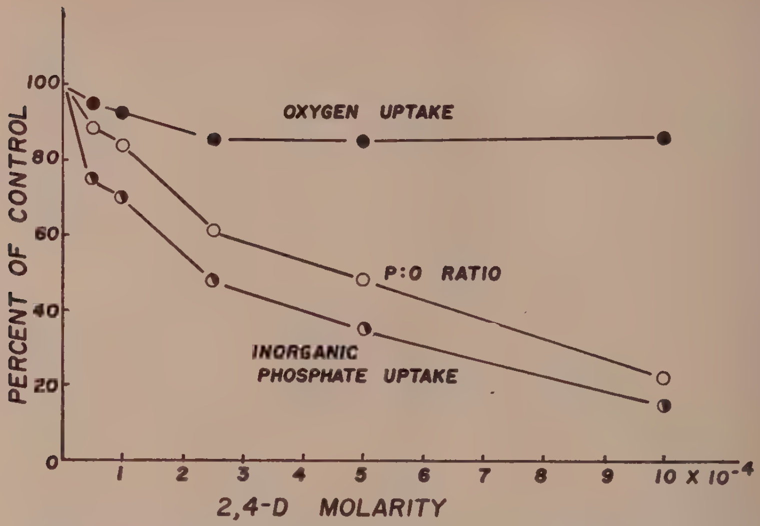

FIG. 1. Effect of 2,4-D on oxidative phosphorylation in rat liver mitochondria. Vessel contents: 1.5×10^{-2} M Na-glycylglycine, pH 7.4, 2.0×10^{-2} M K-phosphate, pH 7.4, 2.8×10^{-2} M glucose, 5.0×10^{-2} M KCl, 8.0×10^{-3} M $MgCl_2$, 2.5×10^{-3} M K-adenosinetriphosphate, 8.3×10^{-6} M cytochrome c, 1.3×10^{-2} M Na-pyruvate, 4.3×10^{-3} M Na-malate, 0.1 ml yeast hexokinase(9), 2,4-D concentration as indicated, 0.4 ml rat liver mitochondria suspension(9), 1.2×10^{-2} M NaF, total volume 2.0 ml, 0.2 ml 2 N KOH in center well with fluted filter paper, 0.2 ml 50% trichloroacetic acid in sidearm. Solutions added in the order listed. Gas phase-air, temperature 20°C. Duration of the experiment 30-40 minutes. Each point is the average of 3 separate experiments, percent activity for any individual vessel being calculated from its respective control. P:O ratios in the controls ranged from 2.34 to 2.44.

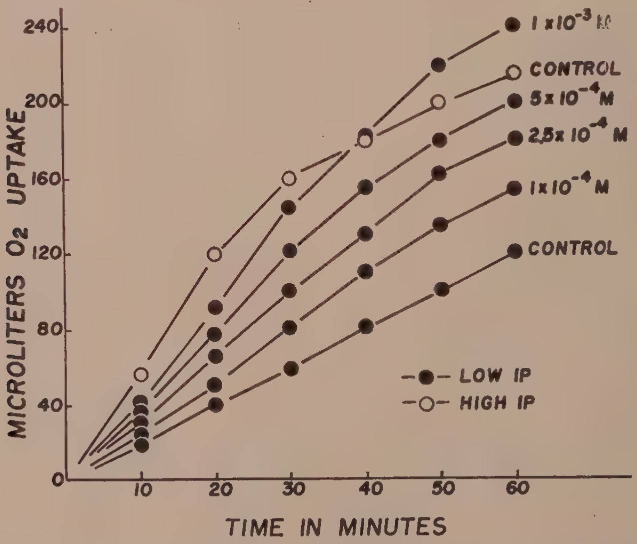

FIG. 2. Effect of 2,4-D on the respiration of rat liver mitochondria in a phosphate deficient system. Vessel contents the same as in Fig. 1, except that the low phosphate vessels contained 5×10^{-4} M K-phosphate, pH 7.4. The difference in ionic strength between low and high phosphate vessels was made up by the addition of KCl to the low phosphate vessels. Temperature 30°C.

TABLE I. Effect of some Plant Growth Substances on Respiration of Rat Liver Mitochondria in a Phosphate-Deficient System.*

Compound	Molar concentration	Oxygen uptake†
High phosphate control		200
Low phosphate control		110
		114
2,4-dichlorophenoxyacetic acid	1×10^{-3}	226
p-chlorophenoxyacetic acid	5×10^{-4}	116
	1×10^{-3}	142
Indole-3-acetic acid	5×10^{-4}	97
	1×10^{-3}	102
Indole-3-butyric acid	5×10^{-4}	103
	1×10^{-3}	100
α-naphthaleneacetic acid	5×10^{-4}	105
	1×10^{-3}	112

* Conditions as in Fig. 1 and 2.

† Microliters per 40 min

of the control level. Uncoupling effects can be seen at concentrations of 2,4-D as low as 5×10^{-5} M.

Since we were able to show that 2,4-D could dissociate oxidative and phosphorylative mechanisms, this compound and other plant growth substances were studied in the phosphate deficient system. The results with 2,4-D are shown in Fig. 2. Increasing the concentrations of the plant growth substance resulted in increasing respiratory rates. Analysis at the end of the run showed that the vessels exhibiting the greatest stimulation also contained the highest amounts of inorganic phosphate. In a typical experiment (Table I), indole-3-acetate, indole-3-butyrate and α-naphthaleneacetate were ineffective in producing respiratory stimulation in concentrations as high as 1×10^{-3} M. While 2,4-D in concentrations of 1×10^{-3} M doubled the rate of the low phosphate system (equalling or exceeding the high phosphate control rate), p-chlorophenoxyacetate produced about a 30% increase in rate above the low phosphate control level (Table I). Thus it was possible by limiting the phosphate concentration to demonstrate a stimulatory effect with 2,4-D and to a lesser extent with p-chlorophenoxyacetate.

Discussion. There is little doubt that the stimulation of the respiration of liver mitochondria observed under the influence of 2,4-D is a result of its uncoupling properties maintaining in the phosphate deficient system an effective level of inorganic phosphate. Whether or not this is the mechanism by which 2,4-D produces stimulation of plant respiration is a question which cannot be answered at this time. A suggestion has been made to the effect that inorganic phosphate might form an ester phosphate link with phenoxyacetic acids which on cyclization of the compound would result in the generation of a high energy bond(11). Plant tissues apparently contain systems for energy transfer analogous to the adenylate systems of animal tissues(6,12) and it is possible that the phenoxyacetic acids, at least, interact with these systems. In a discussion of this sort it must be remembered that there are at least 2 ways in which respiration of intact tissues can be stimulated: 1. An increased demand on the energy supplying sources by the requirements of increased growth or function, or 2. An uncoupling action disrupting the control over respiration which is ordinarily mediated by the level of inorganic phosphate and acceptors therefor. Conceivably plant growth substances might act upon either or both of these postulated mechanisms depending upon the concentration of the substance present at any given time either *in vitro* or *in vivo*. Whatever the relation of the uncoupling effect to plant metabolism, 2,4-D may perhaps prove to be a useful tool for the investigation of the coupling mechanisms of tissues in general.

Summary. 2,4-dichlorophenoxyacetic acid was shown to be an uncoupling agent for oxidative phosphorylation and to stimulate the respiration of rat liver mitochondria in a phosphate-deficient medium. p-chlorophenoxyacetic acid was much less potent in producing the above effects. Indole-3-acetic acid, indole butyric acid, and α-naphthaleneacetic acid were inactive. The results are discussed in relation to the metabolism of plant and animal tissues.

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Placental Transfer of Radioactive Digitoxin in Rats and Guinea Pigs.* (19682)

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The placental transfer of digitalis has received little or no attention in the past. Since the availability of radioactive digitoxin, studies on the penetration of digitoxin across the placental membrane in laboratory animals have become possible.

Method. Rats and guinea pigs were selected as test animals since their placenta is in some respects morphologically similar to that of man(1). Uniformly labeled C^{14}-digitoxin prepared by biosynthesis(2) and having a specific activity of 620,000 counts per minute per milligram (0.43 μc per mg) was administered intravenously to each animal. The dose used for each Sprague-Dawley rat was 0.175 mg (approximately 0.5 μg/g) and for each guinea pig 0.25 mg (approximately 0.25 μg/g).

The animals were sacrificed one hour after intravenous administration of the drug and the fetuses and maternal hearts were removed. In addition the guinea pig fetal hearts were isolated. The tissues were thoroughly washed in isotonic saline to remove blood, then dried between filter paper, weighed, and homogenized in Potter and Elvehjem homogenizers with 50% ethanol. Guinea pig fetuses (minus the hearts) were prehomogenized in a Waring blender and an aliquot transferred to Potter and Elvehjem homogenizers. The fetal hearts of the rats were not isolated because of their small size.

The extraction procedure employed for the isolation of unchanged digitoxin and fractionation of its metabolic products consisted of diluting the homogenates with 50% ethanol, adding a few milligrams of non-radioactive digitoxin as carrier, and making a liquid-liquid extraction with chloroform(2). The chloroform fraction was evaporated to dryness, taken up in 50% methanol, extracted with carbon tetrachloride to remove fats and lipids, and re-extracted with chloroform. The latter chloroform fraction was evaporated to dryness, again taken up in chloroform (C.P.), and then allowed to pass through an alumina column. Successive elutions were made with the following solvents: 1% ethanol in chloroform, 10% ethanol in chloroform, and 40% aqueous ethanol. Unchanged digitoxin was found to occur in the 10% ethanol in chloroform eluate. Identification of the drug was accomplished using paper partition chromatography(2), polarographic analysis(3), and color reaction tests(2). All radioactive samples were analyzed using an internal gas-flow Geiger counter(4). The maximum counting error was 10% with the error for most counting samples being less than 5%. The various samples showing radioactivity other than unchanged digitoxin were considered to contain metabolic products of the parent drug. The

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† U. S. Public Health Service Fellow.

TABLE I. Placental Transfer of C^{14} Digitoxin in Pregnant Guinea Pigs.

Pregnant guinea pigs*	Approximate period of pregnancy (term = 1)	Fetus				Total C^{14} in fetus
		Unchanged digitoxin		Metabolites		
		% original dose injected	% of total C^{14} in fetus	% original dose injected	% of total C^{14} in fetus	% original dose injected
G.P. 1	.40	.28	1.6	17.3	98.4	17.58
2	.70	.55	2.1	26.4	97.9	26.95
3	.90	.35	1.7	20.05	98.3	20.40
4	.95	.73	3.0	23.70	97.0	24.43
Avg	.74	.48	2.1	21.9	97.9	22.34
Stand. dev.		±.10	±.32	±1.9	±.32	±2.1

* Animals sacrificed one hr after administration of drugs.
Amt. C^{14} digitoxin/G.P. = 0.25 mg; Sp. Activity = 620 cpm/μg; route administration = I.V.

TABLE II. Placental Transfer of C^{14} Digitoxin in Pregnant Rats.

Pregnant rats*	Approximate period of pregnancy (term = 1)	Fetus				Total C^{14} in fetus
		Unchanged digitoxin		Metabolites		
		% original dose injected	% of total C^{14} in fetus	% original dose injected	% of total C^{14} in fetus	% original dose injected
1	.90	.05	5.	1.03	95.0	1.08
2	.80	.03	8.2	.39	91.8	.43
3	.50	.02	6.2	.35	93.8	.37
4	.85	.04	6.1	.69	93.9	.73
Avg	.76	.04	6.4	.61	93.6	.65
Stand. dev.		±.007	±.66	±.16	±.76	±.03

* Animals sacrificed one hr after administration of drugs.
Amt. C^{14} digitoxin/rat = 0.175 μg; Sp. activity = 620 cpm/μg; route administration = I.V.

nature of these metabolic products is not known at the present time.

Results and discussion. The results listed in Tables I and II indicate that in all instances digitoxin crosses the placenta of guinea pigs and rats. Although guinea pigs and rats have morphologically similar fetal membranes, the data indicate that in the guinea pig digitoxin and possibly its metabolic products are able to cross the placenta much more readily than in rats. One hour after administration of digitoxin the average placental transfer for guinea pigs was 22.3 ± 2.1% of the original dose as compared with 0.65 ± .03% for rats.

Less than 9% of the carbon 14 found in the fetuses of both species represented unchanged digitoxin, while the rest was in the form of its metabolites. The metabolite-digitoxin ratio in the fetal guinea pig is approximately 47-fold, while in the fetal rat it is approximately 15-fold. Fischer and coworkers (5) have shown that one hour after administration of radio-digitoxin to normal rats, the major portions of the radioactivity in the organism was in the form of unchanged digitoxin. Therefore, the extremely high metabolite-digitoxin ratio in the fetus may be due either to a high conversion rate of the drug by the embryonic tissue or to a selective penetration of the metabolites across the placental barrier, or both.

Although the present study was not designed to determine at which period of gestation maximum rate of placental transfer was effected, our data suggest that as the gestation period increases there is a corresponding increase in the rate of transfer of digitoxin across the placenta.

To determine whether there is an uptake of digitoxin and its metabolites in the embryonic heart, fetal hearts of guinea pigs were assayed and compared with the maternal heart. The data shown in Table III indicate that embryonic hearts from 3 of the guinea pigs contained unchanged digitoxin, while embryonic hearts from all of the guinea pigs contained its metabolic products. Although on an organ basis the embryonic hearts accumulated less of the drug than the maternal heart, on a weight basis it is seen that the fetal heart has a higher concentration. The latter fact is interesting for it suggests the possibility of digi-

TABLE III. Comparison Between Fetal and Maternal Heart on Uptake of C^{14} Digitoxin and its Metabolites in Guinea Pigs.

	Unchanged digitoxin				Metabolites			
	Fetal hearts		Maternal heart		Fetal hearts		Maternal heart	
Animal*	μg/heart	μg/g	μg/heart	μ/g	μg equiv†/ heart	μg equiv/ g	μg equiv/ heart	μg equiv/ g
G.P. 1	N.S.C.‡	——	.03	.01	.06	.46	.23	.09
2	.05	.09	.03	.01	.07	.12	.45	.16
3	.03	.07	.02	.01	.09	.21	.28	.18
4	.02	.03	.02	.01	.05	.06	.19	.10
Avg	.03	.06	.03	.01	.07	.21	.29	.13
Stand. dev.	±.007	±.013	±.004	±.0	±.006	±.09	±.003	±.08

* Animals sacrificed one hr after administration of drug.
† Amount of original drug converted into metabolic products.
‡ No significant counts.

talizing an embryonic heart beyond the therapeutic level.

Summary. 1. Digitoxin, and possibly its metabolic products, crosses the placenta of rats and guinea pigs. 2. Larger amounts of both unchanged digitoxin and its metabolites were found in the fetuses of guinea pigs than in the fetuses of rats. 3. Embryonic tissue may have a marked ability to catabolize the drug, or there may be a selective penetration of the metabolites across the placenta, as noted by its high metabolite-digitoxin ratio. 4. On a tissue weight basis, digitoxin and its metabolites were found in higher concentrations in the embryonic heart than in the maternal heart.

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Factors Influencing Development of Hypophyseal Tumors in Mice After Treatment with Radioactive Iodine.* (19683)

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It has been shown(1) that quantities of radioiodine of approximately 200 μc given to mice destroy, or almost destroy, the thyroid gland. This treatment is followed after an interval of about a year by development of fatal tumors of the pituitary gland. Gonadectomy neither favors nor inhibits the growth of the pituitary tumors(2).

In further exploration of the reasons for the tumorous growth, the following 2 series of experiments were initiated.

When C_{57} mice are fed the Remington "low-iodine" diet for one or 2 weeks before they are given I^{131}, 60% of the radioiodine accumulates in the thyroid gland. With such use of the Remington diet it is possible to produce the same thyroidal destruction with 30 μc of I^{131} as is produced in mice fed normal (Purina chow) diets by 200 μc(2) of I^{131}. The systemic, or "whole-body" radiation in the 2 cases is, however, very different,

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TABLE I. Relationship Between Dose of I^{131}, Dietary Regimen, and Incidence of Late Hypophyseal Tumors.

Group	No. of mice	Pre-I^{131} diet*	Dose of I^{131}, μc	Age at time sacrificed (mo)	Wt of pituitaries		Condition of thyroid gland
					In detail, mg	Avg, mg	
1	10	Purina Chow	None	12 –17	.7, .7, .9, .9, .9, 1, 1.2, 1.4, 1.6, 1.7	1.2	Normal
2	5	"	30	12.5–16.5	1.1, 1.3, 1.4, 1.6, 2.8	1.8	Normal or slight medullary fibrosis
3	14	"	200	11.5–16.5	2.6, 6.2, 12.4, 12.9, 17.2, 25.8, 32.6, 37, 52.1, 91.1, 98, 110.4, 116.2	48.9	Complete, or almost complete, destruction
4	9	Low-iodine	30	11 –16	.7, 1, 1, 1.1, 1.1, 1.4, 1.7, 1.8, 3.8	1.5	Complete, or almost complete, destruction
5	12	"	200	11 –16.3	11.2, 11.8, 13.6, 14.3, 14.8, 16.2, 18.6, 24, 36.4, 37.4, 56.2, 110	30.4	Complete destruction

* The low-iodine diet was begun at the age of 44-45 days, and fed for 35 days. The 30 μc or 200 μc doses of I^{131} were inj. subcut. on the tenth day of feeding the special diet.

varying by a factor of about 15 times. For the purposes of this experiment 24 C_{57} strain mice were fed the Remington diet[†] for 35 days. Radioiodine was given subcutaneously on the 10th day of this period in doses of 30 μc, or 200 μc (Table I). An equal number of mice was given these dosages of I^{131} while on a regimen of Purina Lab Chow.

In a second series of C_{57} female mice (Table II) all were given the pituitary tumor-eliciting dose of 200 μc of I^{131}. Of this group 25 then were given 2 subcutaneous injections per week of 2.5 μg of d-l thyroxine for the duration of their lives. In this same series, 17 mice were implanted subcutaneously 25 days after the I^{131} treatment with 2 complete lobes of thyroid glands from normal mice of the same age (70 days) and strain (C_{57}). In a similar way, 7 mice were implanted with an ovary, 7 with a pituitary, and 9 with an adrenal gland (Table II).

Results and discussion. The data summarized in Table I indicate that radiothyroidectomy *per se* will not lead to production of hypophyseal tumors. The mice whose thyroids were destroyed by 30 μc of I^{131} developed no tumorous pituitaries after periods of more than a year. This does not seem to be due to any inhibitory effect of the low-iodine diet because this diet did not prevent growth of pituitary tumors in mice treated with 200 μc of I^{131}.

In the doses used, thyroxine injections reduced the incidence and size of the pituitary tumors expected to develop after I^{131} treatment (Table II). It might be that higher doses of thyroxine would have been even more effective. This confirms the report of Goldberg and Chaikoff(4), and would seem to support their contention that the abnormal pituitary growth is due to the hypothyroidism resulting from the radiant destruction of the thyroid gland. However, the absence of pituitary tumors in the "30 μc-low iodine diet" group of mice indicates that such an uncomplicated conclusion is not possible.

The most effective agent found in these experiments in prevention of the pituitary tumorous growth was implantation of new thyroid tissue. Only in the few instances in which the implanted thyroids failed to survive were there pituitary tumors (Table II). No such protective action was obtained from implants of pituitary, adrenal, or ovarian tissue. The pituitary transplants completely degen-

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[†] The ingredients for this diet were as follows (expressed as percent by weight): yellow corn meal 73.5%, wheat gluten 19.0%, dry brewer's yeast 5.5%, iodine-free NaCl 1.0%, $CaCO_3$ 1.0%. The NaCl was purified by precipitation from ethyl alcohol according to the procedure suggested by Pinching and Bates(3).

TABLE II. Relationship of Treatment of Mice with Thyroxine or Endocrine Gland Transplants to Development of Hypophyseal Tumors after Injection with I^{131}.* (All mice in this table were injected with 200 μc of I^{131} at 45 to 60 days of age.)

				No. of mice in which implant was		Wt of pituitaries	
Group	No. of mice	Nature of treatment†	Age at time of sacrifice (mo)	Re-covered	Not re-covered	In detail, mg	Avg, mg
1	25	Thyroxine inj., 5 μg/wk	9 –10	—	—	1.6, 1.8, 2, 2, 2	1.9
			14 –16.5	—	—	1.8, 2.2, 2.5, 2.8, 2.8, 2.9, 3.2, 3.4, 3.6, 3.6, 3.6, 3.8, 4, 5.6, 9, 9.3, 10, 13.4, 13.9, 28	6.5
2	17	Implant of thyroid, 2 lobes	12.5–16.5		4	6, 66.4, 94.2, 138.3	76.2
				13		.4, .8, .9, 1, 1.6, 1.8, 2, 2.4, 2.6, 2.8, 3.4, 4, 4.8	2.3
3	7	Implant of one ovary	12 –15		1	35.6	33.7
				6		4.5, 10, 11.9, 35.8, 45.6, 92.6	
4	7	Implant of one pituitary	12.5–15.3	7		26.2, 55.4, 70.6, 77.8, 126.2, 153, 157.4	95.2
5	9	Implant of one adrenal	12 –15		1	18	29.7
				8		9.4, 19.4, 22.8, 29.9, 38.1, 39.2, 49, 70.8	

* For additional control data see groups 1 and 3 in Table I.

† Thyroxine inj. were begun directly after the I^{131} inj. The tissue implants all were made 25 days after the I^{131} inj.

erated; the few surviving adrenals contained only a bit of the capsule and small amounts of zona glomerulosa; most of the ovarian transplants were recovered in an involuted condition and they lacked most of their germinal epithelia.

Summary. 1. Radiation thyroidectomy (under specified dietary conditions) with smaller amounts of I^{131} is not followed by pituitary tumors in C_{57} mice. 2. Such radiation thyroidectomy with larger quantities of I^{131} is followed by pituitary tumors. 3. Thyroid implants or adequate thyroxine dosages prevent appearance of the pituitary tumors after suitable I^{131} treatment. 4. Similar implantation of pituitary, ovarian, or adrenal tissue in treated mice does not prevent growth of the tumor. It is concluded that the radiation thyroidectomy is a necessary, but not the sole factor, leading to growth of the pituitary tumor in I^{131}-treated mice. It appears likely that another factor, related to the ionizing radiation itself, may be an influence aggravating or synergizing the thyroidectomy in its tumorigenic action on the pituitary gland of the mouse.

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Blood-Clotting Studies on Dogs Internally Irradiated with Radio-Gold.* (19684)

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Radio-gold (Au^{198}) was administered intravenously as colloidal sol to 5 adult dogs in single dose approximating 20, 10, 10, 5, and 1.6 millicuries per kg, respectively. Two dogs received stable colloidal gold in volumes, per kg of body weight, comparable to the 10 millicuries per kg radio-gold experiments. An untreated control animal was kept with the irradiated dogs in an isolation hut and at no time showed any abnormal findings. Prior to the injections all animals were apparently healthy and gave normal prothrombin consumption tests. The dogs were observed carefully throughout the experimental period and autopsy findings were supplemented by extensive histopathological studies, details of which will be reported elsewhere. During life they were subjected to repeated hematological observations, including routine platelet counts. Special blood-clotting studies were made as described, with particular emphasis upon "prothrombin consumption tests" by both the 2-stage and modified one-stage methods. Whole blood clotting-times at 37°C were noted in conjunction with these tests and incidental observations were made on clot retraction both in 1-1½ hours and after 24 hours. Fibrinolysis was also looked for at the end of 24 hours (at 37°C) but was invariably absent. Icteric sera were particularly noted and observed in the later stages in dogs receiving 10-20 millicuries per kg.

Methods. Prothrombin consumption tests, etc. Jugular vein blood was secured with needle and syringe, starting a timer at midpoint of bleeding. 2.7 ml blood was placed in each of 7 marked tubes. Tube 1, containing 0.3 ml of 0.1 M sodium oxalate, was shaken and immediately centrifuged (3 min. at 3000 r.p.m.) for plasma. The other tubes were placed in a water bath at 37°C and observed for *whole blood clotting-time* (c.t.). *Clot-retraction* was noted in remaining tubes at 1-1½ hours and the last tube reobserved at 24 hours. *Clot-lysis* was also looked for, but was invariably absent. Tubes 2-6 were oxalated, respectively, at 6, 15, 30, 60 + c.t., and 90 minutes after bleeding (timer zero), loosening the clot with a glass rod and centrifuging for serum. *Thrombin test.* 0.1 ml serum (or plasma) + 0.2 ml fibrinogen, at 37°C, showed no thrombin in the plasma and very little in any of the sera, clotting-times usually being over an hour. *One-stage (modified) prothrombin clotting-time (PCT) test* (1). Mixtures of 0.1 ml each of test serum (or plasma), $BaSO_4$-adsorbed normal dog plasma (to supply fibrinogen), thromboplastin (Schieffelin's "soluplastin"), and 0.04 M $CaCl_2$ were timed for clotting at 37°C. *Two-stage prothrombin assay.* The Iowa method, as modified by Ware and Seegers(2), was closely followed, with the very minor substitutions of (a) 10 unit/ml Upjohn's bovine thrombin, for defibrination (1st step), required for plasma and 6 minutes serum only; (b) choice of either 1) Difco incubation mix (courtesy Dr. C. W. Christensen) or 2) mixture of 10.6 ml saline (0.85% NaCl), one ml imidazole buffer (pH = 7.3), 4 ml 15% *purified* acacia, 0.6 ml thromboplastin (Schieffelin's "soluplastin"), 0.9 ml 0.1 M $CaCl_2$; (c) dog fibrinogen, prepared by adsorbing normal oxalated plasma with $BaSO_4$, precipitating with one-fourth saturated $(NH_4)_2SO_4$, and dialyzing against saline containing 1:200 of 38% trisodium citrate. Tests were made at 28°C and units per ml computed from total dilution multiplied by factor (from Seegers' table) to correct for clotting-times other than 15 seconds. *Test for circulating anticoagulant, etc.* Clotting-times, at 37°C,

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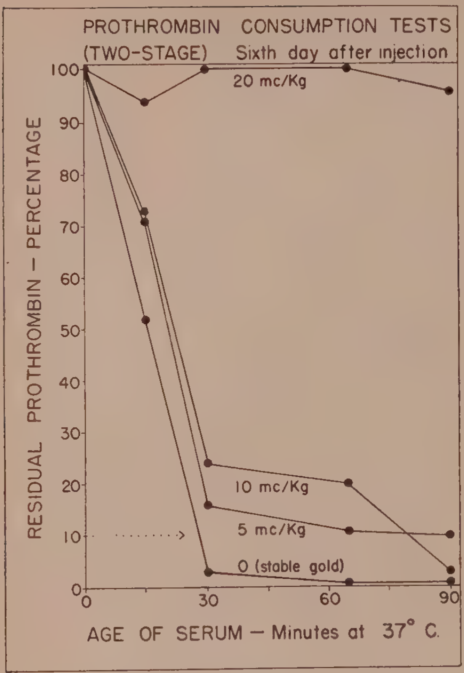

FIG. 1. Prothrombin consumption tests (2-stage). Sixth day after inj.

of mixtures of (a) normal dog plasma: 0.2, 0.15, 0.1, 0.05, 0.02, 0.01, 0; (b) test plasma: 0, 0.05, 0.1, 0.15, 0.18, 0.19, 0.2, respectively, recalcified with 0.2 ml 0.02 M $CaCl_2$. A heparin-antithrombin type of circulating anticoagulant would increase clotting-times at the start of the series, whereas serious clotting defects (*e.g.*, hemophilia, severe hypoprothrombinemia, or lack of accelerin or proconvertin) would give longer clotting-times in the later mixtures.

Prothrombin consumption tests: 2-stage method. Fig. 1 shows the results of tests made on the 6th day after the gold injection. The residual prothrombin, assayed by the "2-stage" method, is recorded, as percentage of the original (*zero* time) plasma unitage, for serum removed after oxalation of consecutive blood samples aged at 37°C, respectively, for 15, 30, 60 + c.t., and 90 minutes after withdrawal from the jugular vein. The control, injected with stable gold, is quite normal, with only negligible amounts of prothrombin remaining after 30 minutes. Anything more than 10% residual prothrombin in the 60-90 minute period is significant evidence of impaired prothrombin utilization. As may be seen, this occurred in the 3 irradiated animals, being extremely marked at the 20 millicuries dosage. These findings were confirmed, somewhat more strikingly, in data obtained subsequently, especially when the effects of the internal irradiation on the prothrombin utilization were maximal (Fig. 2).

Fig. 3 shows, for all test animals, the course of the phenomenon, as indicated in the data of the 90-minute tests of observations repeated at intervals throughout the study period. Significant observations are summarized as follows: A. The 2 animals receiving stable gold remained normal, showing mere traces of residual prothrombin, in observations over a 2-week period. Sacrificed 5 months later, their livers were normal. B. One animal received a token dose of 1.6 millicuries/kg of radiogold. Its tests remain below the significant 10% level. Sacrificed on the 106th day, its liver and other organs were normal.

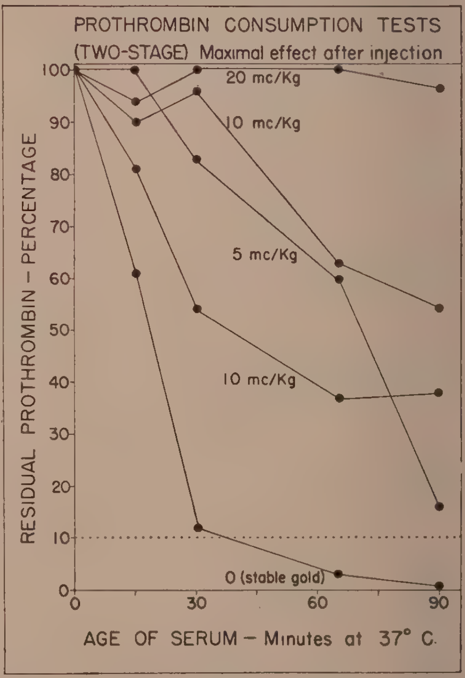

FIG. 2. Prothrombin consumption tests (2-stage). Max. effect after inj.

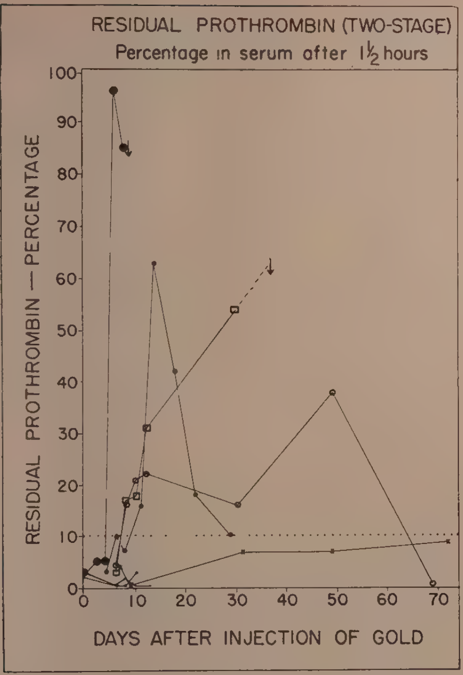

FIG. 3. Residual prothrombin (2-stage). % in serum after 1½ hr (see text). Arrow indicates death of dog.

C. The dog receiving the 5 millicuries dose showed positive tests on the 6th day, increasingly so until the 18th day (a severe reaction) and return toward normal by the 29th day. It is surviving, now 3 months later. D. In one animal receiving 10 millicuries per kg, the test became positive on the 6th day, more so through the 12th day, fluctuated in severity at the 30th and 49th day, and was back to normal on the 69th day. Marked jaundice was noted through the second month but lessened, and death on the 83rd day was unexpected. Autopsy, however, revealed marked liver damage reported as "chronic cirrhosis, with atrophy of liver cords, engorged sinusoids, and considerable hemosiderin, apparently from old hemorrhage." E. In the other animal receiving 10 millicuries radiogold, the course during the first 10 days was very similar to the foregoing. Thereafter, the prothrombin consumption test data, icterus, and clinical conditions were more severe and progressive and the animal died on the 37th day. In addition to widespread (particularly pulmonary) hemorrhages, typical of the radiation syndrome, the autopsy showed acute liver damage reported as "massive and complete hemorrhagic necrosis." F. In the last animal receiving 20 millicuries radiogold, the prothrombin tests (as in the other cases) were normal before the 6th day. There then occurred an almost complete failure of prothrombin utilization. Severe jaundice was noted at this time and death occurred on the 9th day, with typical radiation syndrome hemorrhages. At autopsy, the liver was markedly hemorrhagic, icteric, and necrotic. A section of duodenum, which nestled under the liver, was swollen by a massive interstitial hemorrhage. There was bleeding into the neck from the simple venipuncture of the previous day. The hemorrhages, stated to be "typical" of the radiation syndrome, as noted in the last 2 animals, included: bleeding into the lungs, petechiae in serous membranes (subendocardial, subepicardial, peritoneal), spotty hemorrhages in mucosa of the alimentary canal, lymph node hemorrhages, and (in the 10 millicuries animal) extensive bleeding into the mesentery and retroperitoneal tissues.

Tests by one-stage method. Fig. 4 summarizes the significant results of prothrombin consumption tests made by the modified *one-stage* method, on the same samples as used for the 2-stage tests. The chart records actual clotting-times, at 37°C, of mixtures of test serum (or plasma), $BaSO_4$-adsorbed normal dog plasma (to supply fibrinogen), thromboplastin, and calcium. The longer the clotting-time, the less the prothrombin. The dotted line, at a value of 10 sec., divides the data into 2 groups. Those that are "normal" in showing values above this level by the 30 min. test period (and thereafter) include: (a) all tests before the gold injections on the 5 test animals, (b) the 2 additional animals (A,B) receiving stable gold; and (c) the animal (X) after receiving only 1.6 millicures/kg of Au^{198}.

The "positive" group includes the 4 animals irradiated at 5-20 millicuries/kg. In their sera, the clotting test values remain well below the 10 sec. "critical level" in observations during the pathological period. Qualitatively, at least, this confirms the findings by the 2-

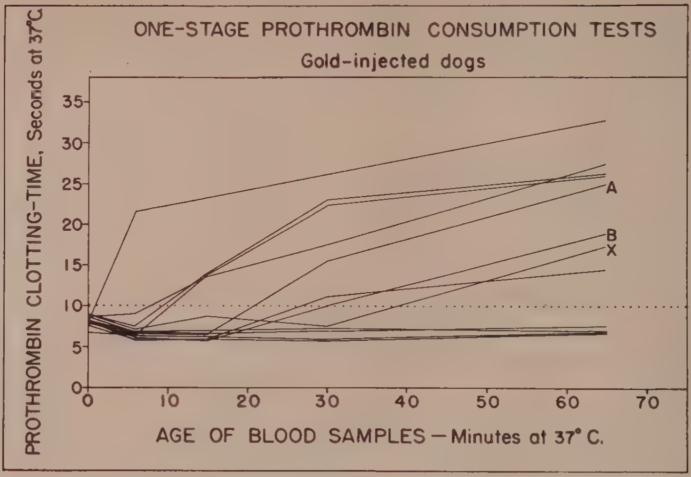

FIG. 4. One-stage prothrombin consumption tests. Gold-inj. dogs (see text).

stage method. Strict quantitation by the one-stage method, however, presents some difficulties. One of the reasons for this is suggested by a finding, as can be seen from the chart, that the PCT values in the earlier tests show a drop, instead of the increase seen later in the "normals." Without discussing this phenomenon, previously reported by others(3), we may state that it seems to be a normal result of the appearance, during blood clotting, of some accelerator-type factor which influences the one-stage test. We would agree that it might be Alexander's SPCA or Owren's convertin(4). It is significant that this appearance is evident in all the irradiated animals' sera, suggesting that they are normal in respect to this factor.

Tests for circulating anticoagulant, etc. On several occasions we tested for the possibility of a circulating anticoagulant by measuring recalcification clotting-times of mixtures of the test animal's plasma and normal plasma. As shown in Table I (first row) there is no significant c.t. difference and hence no evidence for circulating anticoagulant or other abnormality which these tests could have revealed. In fact, with the use of an older "normal" plasma in the second series of tests, the test plasma lessened clotting-times toward the usual value from which the particular normal dog plasma had deviated, owing, in all probability, to loss of accelerin or labile factor(4). Penick(5) has reported that the antihemophilic factor remains normal in X-irradiated dogs and his and Cronkite's radiation studies (4,6) cover many of the points we have mentioned.

TABLE I. Test for Circulating Anticoagulant, etc. Plasma-recalcification clotting-times, seconds at 37°C. Dog receiving 10 mc per kilo radio-gold (Au^{198}).

% test plasma	0	25	50	75	90	95	100
% normal "	100	75	50	25	10	5	0
10th day after inj.	55	56	57	56.3	61	59	63
12th " " "	123	73.5	62.5	63.8	60.3	62	59.3

Other data. In our internally irradiated animal plasmas, there was no hypoprothrombinemia. Whole blood clotting-times were all normal except for one test (on the 6th day) in the 20 millicuries/kg dog, when it was about twice normal. Clot-retraction was definitely lessened in the 6-8 day period on this 20 millicuries animal, and in the 10-30 and 10-40 day periods on the two 10 millicuries animals.

Platelets. Our platelet counts, made by a good hospital technician, while perhaps not up to the highest research standards, give acceptable data which are shown in Table II. There seems to be some lack of correlation with the results of the 2-stage prothrombin tests, particularly in the appearance of "positive" tests in 2 animals on the 6th day with

TABLE II. Comparison of Platelet Counts (A)* and Residual Prothrombin (B)† in Dogs Injected with Radio-Gold.

	Dosage of radio-gold					
	20 mc/kg		10 mc/kg		5 mc/kg	
Day after inj.	(A) × 100	(B) %	(A) × 100	(B) %	(A) × 100	(B) %
0	3545	4	2384	—	3125	—
2	2175	7	7650	—	—	—
4	2425	5	—	—	3375	5
6	450	100	3396	20	3725	11
8	225	86	1394	41	1350	3
10	(Died 9th day)		2190	27	—	60
13			260	88	775	67
18			131	63	1450	34

* (A) Platelet counts: per mm³.

† (B) Residual prothrombin: % left in 60 min + clotting-time, by 2-stage test.

normal platelet levels and divergent results on the 8th day, when both animals showed about the same degree of thrombocytopenia. In later tests the severe thrombocytopenia and markedly "positive" prothrombin consumption tests do go together. We accord much respect to the cited data of Cronkite and of Penick on this topic. It is certainly true, and we have data from our own laboratory to prove it, that platelets are essential for plasma clotting, normally. They do act, however, in conjunction with the plasma factors and I believe we need to investigate the latter more fully before concluding that the defect of prothrombin consumption in radiation sickness (resembling that seen in thrombocytopenia(7) and in hemophilia(3)) is solely due to reduction in the number or quality of the blood platelets.

Summary. Internal irradiation with radiogold (Au^{198}), in sufficient dosage, and after several days' latency, induces a blood coagulation defect of which the prothrombin consumption test is a sensitive index. By using this test through the course of the irradiation, the 2-stage values seem to reflect, with some accuracy, the severity and probable outcome of the radiation injury. Similar data are becoming available in several animal species (including man) and with various types of irradiation. We have begun to collect data on human cancer patients under isotope radiation therapy in order to learn more about this interesting phenomenon.

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Rickettsialpox. II. Recovery of *Rickettsia akari* from mites, *Allodermanyssus sanguineus,* from West Hartford, Conn.*† (19685)

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During December, 1951, and January, 1952, 7 cases of rickettsialpox occurred in persons exposed in an apartment house located in West Hartford, Conn.(1). Samples of mites collected by Dr. M. E. Rindge from the walls of incinerators in the basement of the building concerned were received in this laboratory through the courtesy of Dr. John R. Paul, Professor of Preventive Medicine, Yale University School of Medicine.‡ The mites arrived in a tightly stoppered screw cap bottle which was packed in solid carbon dioxide. It is the purpose of the present paper to report the recovery and characterization of *Rickettsia akari* from these frozen mites by two different methods.

Materials and Methods. The laboratory procedures used for the primary recovery of this strain in mice and in developing chick embryos differed in no important respects from those previously described(2). These involved (a) preparation of a suspension of mites ground in sucrose PG(3); (b) inoculation of mice with portions of this suspension by the intra-abdominal route; and (c) injection of the yolk sacs of developing chick embryos with mite suspension, treated with antibiotics to suppress the growth of bacterial contaminants presumably present upon the surfaces of the mites. The criteria for the recognition of *R. akari* which were used in our previous publication(2) were followed in this investigation.

Observations. After the vial containing mites had been quickly thawed at 37°C, it was chilled in an iced bath. Three mites were mounted and identified by one of us (H.S.F.) as *Allodermanyssus sanguineus* (Hirst), and additional specimens were preserved for future reference. The remaining 20 to 25 mites, mainly females, were ground in chilled sucrose PG. A portion of the resulting suspension was used for inoculation of mice, the remainder being shell frozen at –72°C (no. 5559) and used subsequently for strain recovery by inoculation of chick embryos.

1. *Recovery by inoculation of albino laboratory mice*: Each of 6 test mice received 0.2 ml of mite suspension by the intra-abdominal route; one of them was sacrificed on the eighth day following inoculation because it appeared to be slightly sick. Rickettsia-like objects were noted in smears of peritoneal exudate and spleen. A suspension of ground liver and spleen in sucrose PG was used for inoculation of several second-passage mice, and for storage at –72°C (no. 5560B). Further serial passages were not performed. Five first passage mice and 4 second passage mice subsequently survived intra-abdominal challenge doses of the MK strain(4) administered 35 and 27 days respectively following injection of the immunizing inocula.

2. *Establishment in chick embryos of the strain recovered in mice*: The strain recovered in mice was passed to chick embryos by the following procedure. On the eighth day following inoculation with mite suspension, an intact, anesthetized, apparently well, first passage mouse was injected intra-abdominally with 2 ml sucrose PG. The liquid was withdrawn, reinjected and again withdrawn to obtain peritoneal washings from an intact mouse

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‡ The following individuals were instrumental in securing the material and data for this study: Dr. M. E. Rindge, and Dr. James C. Hart (Director), Bureau of Preventable Diseases, and Dr. F. L. Mickle, Conn. State Dept. of Health; Dr. C. E. McPartland, West Hartford, and Dr. E. Nichols of Hartford.

under aseptic conditions. The material in the syringe was used for inoculation of seven-day-old eggs, the yolk sac of each of 7 developing embryos receiving 0.25 ml peritoneal washings. Rickettsia-like objects were seen in smears of each of the yolk sacs of embryos found dead on the sixth and seventh days following inoculation, as well as in each of the yolk sacs of 3 living embryos sacrificed on the seventh day. These infected yolk sacs were pooled, ground with alundum, diluted to 50% with sucrose PG, and centrifuged for 5 minutes at 900 RPM to facilitate separation of gross particles and yolk cake. The resulting suspension (no. 5568) was used for (1) inoculation of 60 eggs from which a yolk sac pool was subsequently made (no. 5564); (2) titration in mice; and (3) subsequent challenge of mice in a reciprocal cross-immunity test with the MK strain. When pool No. 5568 was titrated at dilutions of 10^{-1} through 10^{-7}, by inoculating 0.25 ml doses by the intra-abdominal route to 10 mice for each dilution, the numbers of survivors on the 15th day were 0, 4, 8, 4, 7, 9 and 9 respectively.

When the survivors were challenged 20 days later with at least 10 certainly fatal doses of the MK strain by the intra-abdominal route, all mice survived, giving evidence of solid immunity through 10^{-7}. Although no immunizing endpoint was obtained, therefore, the results indicated the presence of at least 10^6 immunizing doses and approximately one certainly fatal dose in each 0.25 ml portion of 10% suspension.

A further point related to the response of mice to *R. akari* concerns the possibility of a toxic effect in mice similar to that produced by concentrated suspensions of *R. prowazeki*, *R. mooseri*, and certain strains of *R. tsutsugamushi* (syn.: *R. orientalis*). Accordingly, groups of mice were inoculated by the intravenous route (tail vein) with 0.25 ml portions of yolk sac pool no. 5564 in the following dilutions: 20, 6, 2, and 0.6%. Inasmuch as the first deaths were observed on the fourth day, and these in the 20% group, it was inferred that no toxic effect had been demonstrated. Organisms failed to grow in broth or blood agar cultures of any of the yolk sac materials.

3. *Recovery of* R. akari *by direct inoculation of chick embryos with mite suspensions*: The original suspension of mites in sucrose PG (no. 5559) was thawed; a 0.25 ml portion was removed, mixed with 0.25 ml normal yolk sac suspension to act as a nutrient, and incubated for 2 hours at 36°C, in order to provide circumstances conducive to division of bacteria presumably present. After this preliminary incubation, 0.25 ml portions of solutions in K7G(3) in penicillin, 200 units per ml, and streptomycin, 200 mcg per ml, were added and the resulting mixture was incubated for 1.5 hours at 36°C. Inoculation of 4 chick embryos was then performed, the yolk sac of each receiving 0.2 ml of incubated suspension, followed shortly by 0.2 ml of a 2% solution of methyl cellulose containing approximately 11 mg of sulfadiazine.

A sick embryo was sacrificed on the seventh day following inoculation; examination of yolk sac smears revealed rickettsia-like objects, but no bacteria were noted, and no growth of organisms was observed in broth or blood agar cultures of the material. When the yolk sacs of the 3 remaining eggs were harvested on the eighth day following inoculation, rickettsia-like objects were seen in smears of one living and 2 dead embryos. Eight mice were inoculated with a 1% suspension of the egg sacrificed on the seventh day; all were found dead on the sixth and seventh days. Examination of smears of peritoneal exudate, and cut surfaces of liver and spleen revealed large numbers of rickettsia-like organisms within mononuclear cells, and the gross findings were consistent with rickettsialpox infection in albino laboratory mice. It was concluded that a rickettsial strain had been recovered from mites by the relatively simple and rapid procedure of direct inoculation of chick embryos.

4. *Specific complement fixing antibodies produced in response to the Hartford strain*: The serologic evidence recorded for human sera in Table I indicates a response of the Hartford patient to a member of the Rocky Mountain spotted fever group of rickettsiae, and when considered in the light of the pathogenicity of the Hartford strain for mice, it provides evidence in favor of rickettsialpox.

5. *Reaction of Hartford yolk sac antigen with MK immune serum to fix complement*: A rickettsial suspension derived from a yolk

TABLE I. Observations on Complement Fixation Reaction in Characterization of the Hartford Strain of *Rickettsia Akari.*

	Highest serum dilution in which complement is fixed by the specific antigen			
	Rickettsialpox		Epidemic typhus	
Serum	MK	Hartford	(soluble)	Q fever
Human				
Hartford, rickettsialpox, 5th wk after onset	1:320	1:640	0	0
MK immune	1:320	1:320	0	0
Q fever immune	0	0	0	1:320
Normal	0	0	0	0
Guinea pig				
MK immune	1:320	1:320	0	0
" "	1:320	1:320	0	0
Epidemic typhus immune	0	0	1:320	0
" " "	0	0	1:640	0
Murine " "	0	0	1:320	0
Normal	0	0	0	0

Tests were performed by the method of the Army Medical Department Research and Graduate School(5), modified by the use of a magnesium buffer as diluent(6). The antigens were used at a constant dilution of 2 units. 0 = negative at 1:10.

sac pool (no. 5564, representing the second egg passage of the strain recovered by direct inoculation of eggs with mite suspension) was used for preparation of antigen by Method 1 of Topping and Shepard(7), as modified by Boyd(8). Results of parallel complement fixation tests in which this antigen and antigen prepared from the MK strain were performed, using various sera, are summarized in Table I. These observations are taken to indicate that antigens prepared from the Hartford and MK strains are indistinguishable in the complement fixation test as performed.

6. *Reciprocal cross immunity in mice between the Hartford and MK strains of* R. akari. The principles and methods used were similar to those described in a previous paper (2) concerned with characterization of strains of *R. akari.* The Hartford mite strain pool (no. 5568) used in immunizing and challenging mice has been described. On the basis of the titration, it was believed that each 0.25 ml portion of 10% suspension would kill mice between the fifth and eighth days following inoculation of non-immune animals by the intra-abdominal route, hence the use of this dose for challenge. It is possible that loss of titer had occurred, inasmuch as 4 of 16 normal mice survived inoculation with this dose. The MK pool (no. 5530) used for immunizing and challenging mice consistently killed animals which received 0.25 ml of 10% suspension by the intra-abdominal route, and data obtained independently indicated that this corresponded to at least 10 certainly fatal doses. The MK immune mice were inoculated with 0.25 ml of a 10^{-5} dilution of this pool as an immunizing inoculum. The mice were challenged on the 20th day following administration of the immunizing doses. Survivors were recorded on the 15th day as indicated in Table II. The results are interpreted as revealing solid and complete reciprocal cross immunity between the Hartford and MK strains as tested in mice. This fact is regarded as the final piece of evidence that the Hartford strain is indistinguishable from *R. akari.*

Discussion. The recovery of a strain of *R. akari* from *Allodermanyssus sanguineus* (Hirst), collected in West Hartford, Conn.,

TABLE II. Summary of Cross Immunity Tests in Mice with Hartford and MK Strains.*

	Challenge strain	
Immunizing strain	Hartford	MK
Hartford	11/12	38/38
MK	14/14	12/12
Normal controls	4/16	0/20

* Ratio: Survivors to total challenged.

is of interest from several standpoints. It establishes an additional locality record for this rickettsial species which had been positively identified previously only in New York and Boston. It is to be expected that additional strain recoveries will extend the known distribution of *R. akari*. The present strain recovery provides evidence that the patients diagnosed as having rickettsialpox were exposed in an environment where the hazard of infection was present. Finally, this strain recovery, indicating the presence of the causative agent in mites collected in a natural environment, provides additional evidence for the vector role of the house mouse mite, the species previously incriminated by the studies of Huebner, Jellison, and Pomerantz(9) in New York.

The recovery of the present strain by inoculation of mice was supplemented by isolation accomplished by direct inoculation of developing chick embryos with mite suspension which has been appropriately treated. This simple method of strain isolation may be applicable to other problems involving recovery of rickettsiae or viruses from potentially infected arthropods.

Summary. The recovery of a strain of *Rickettsia akari* from mouse mites, *Allodermanyssus sanguineus* (Hirst), collected in West Hartford, Connecticut, is described. The evidence for its identification as *R. akari* is presented, and certain implications of these findings are discussed.

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Effect of Glucose on Oxygen Consumption and Respiratory Quotient of Normal and Denervated Muscle.* (19686)

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The oxygen consumption of denervated muscle was first studied by Langley and Itagki(1). They compared the oxygen content of arterial and venous blood from innervated and denervated extremities of anesthetized cats. Since that time several workers have studied the oxygen consumption of denervated muscle *in vivo*(2,3) and *in vitro* (4,5). These various reports are not in agreement with each other as to the extent of the increase in oxygen consumption by the denervated muscle. Changes in various respiratory enzyme systems following denervation have been reported by Knowlton and Hines (4) and Humoller *et al.*(6,7). The experiments upon which this report is based were done with the hope of clarifying the effect of denervation on the oxygen consumption and respiratory quotient of muscle tissues. The influence of glucose in the perfusate on the oxygen consumption and the respiratory quotient of muscle was also investigated.

Method. One hundred young white rats weighing between 100 and 120 g were used. Unilateral phrenectomy was performed under ether anesthesia by destroying one phrenic nerve in the neck. Ten to 14 days after sec-

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* This investigation was made possible by a grant from Geigy Co,, Inc.

TABLE I. Average Oxygen Consumption and Respiratory Quotient (with standard errors) of Muscle. The intervals represent half hour periods.

	With glucose		Without glucose	
	Normal	Denervated	Normal	Denervated
Q O_2 (1st interval)	8.39 ± .18	8.18 ± .17	6.31 ± .18	7.73 ± .23
Q O_2 (2nd interval)	7.36 ± .19	7.21 ± .17	5.53 ± .16	6.87 ± .22
Q O_2 (3rd interval)	6.61 ± .19	6.42 ± .18	5.05 ± .16	6.16 ± .21
R. Q. (1 hr)	.94 ± .05	.93 ± .04	.95 ± .05	.93 ± .06

tioning the nerve, the animals were sacrificed by cerebral concussion, the majority of them at 11 days. The domes of the diaphragms were placed in Warburg vessels within 10 minutes after the animals were killed. The oxygen consumption was measured by the Warburg manometric method. Krebs-Ringer's solution with phosphate buffer was used. In 96 experiments involving normal muscle and an equal number for denervated muscle, no glucose was present in the solution. In 64 experiments with each type of muscle the solution contained 0.2% glucose. Oxygen was allowed to pass through the flasks for 10 minutes. The flasks were shaken at a rate of 110 per minute in a water bath at 38°C. Before the initial manometric reading, a 15-minute period was allowed for stabilization. Readings were made at 30-minute intervals. The oxygen consumption was calculated from the tissue dry weight. The respiratory quotient was measured by the Dickens and Simer second method according to the procedure described by Dixon(8). Thirteen experiments were performed on denervated and normal muscle without glucose, and 7 with glucose present in the medium.

Results. The averages and standard errors of the oxygen consumption and respiratory quotient determinations are presented in Table I. The oxygen consumption of normal muscle in the absence of glucose is significantly lower (20%) than that of normal muscle with glucose and that of denervated muscle without glucose. A parallel decrease in the oxygen consumption for the 4 muscle-glucose combinations over the 1½-hour period is seen when these results are expressed graphically. No statistically significant difference was observed in the values obtained for the respiratory quotient of the various muscle-glucose combinations.

Discussion. The presence of glucose produced a significant difference in the oxygen consumption of normal muscle, but no significant difference in the oxygen consumption of denervated muscle. Also no significant difference occurred in the oxygen consumption of normal and denervated muscle in the presence of glucose when the oxygen consumption was determined on a dry weight basis. This suggests that in the absence of glucose it is possible for the oxygen consumption of normal muscle to be reduced, whereas it is not possible to reduce that of denervated muscle. Such a loss of the ability of muscle to respond to a reduction in glucose content of the surrounding medium may express a change in the enzyme system of the muscle after denervation.

Assuming that the oxygen consumption of muscle and the dry weight of the muscle decrease proportionately after denervation without change in the total number of cells, on a cellular basis the oxygen consumption of normal and denervated muscle in the absence of glucose should show no significant difference. Then the hypothesis that the enzyme system of normal muscle is able to utilize oxygen to a greater extent in the presence of glucose than can denervated muscle, becomes tenable.

The theory that the normal muscle metabolism is more adaptable to increases in glucose content of the medium seems as logical as the one that denervated muscle metabolism is less adaptable to decreases in glucose. Since there is no significant difference in the respiratory quotient of the glucose muscle combination we may assume that the difference in the oxygen consumption is not the result of change in the metabolic pattern.

Summary. 1. The oxygen consumption of innervated muscle *in vitro* is significantly greater in the presence of glucose than in the absence of glucose. 2. The presence or absence of glucose produces no significant difference in the oxygen consumption of denervated muscle *in vitro*. 3. No statistically significant difference in the respiratory quotients of innervated and denervated muscle with and without glucose in the medium was found.

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Microdetermination of Carbon Dioxide Content of Serum or Plasma with a Glass Electrode. (19687)

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The estimation of carbon dioxide content of serum by titration(1) has been little used in the past, chiefly because gasometric estimation is usually more convenient. Titrimetric micromethods(2), however, have been adopted recently. It occurred to us that the titration curves of different sera would be quite similar in the region of pH 5 to 7 and would enable one to estimate the carbon dioxide content from the pH after the addition of a known amount of acid to low pH, followed by a known amount of alkali to this region of pH. In the present paper we describe a rapid method which has been developed for the determination of carbon dioxide in this way on samples of 0.1 and 0.2 ml of serum or plasma.

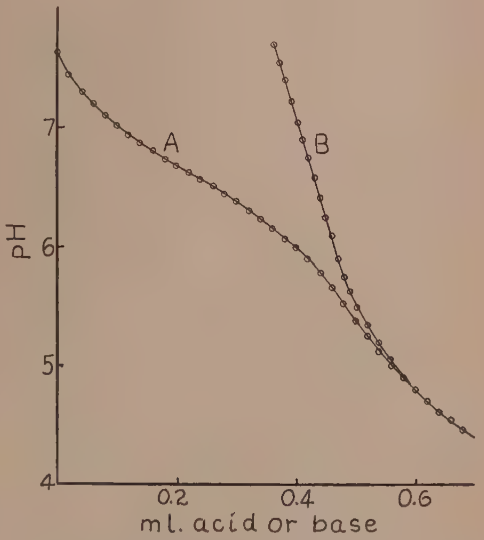

FIG. 1. Representative curve for titration of 2 ml of plasma with 0.2 N hydrochloride and 0.2 N sodium hydroxide.

Experimental. Titration curves were determined in 2 ml samples of serum or plasma obtained under oil from patients in various states of acid-base balance. The samples were diluted with 0.9% saline, and the pH was determined with a Cambridge Research model glass electrode after interval additions of about 0.02 ml of standard 0.2 N hydrochloric acid until the pH was in the region of 4.0. Then standard 0.2 N sodium hydroxide was added in the same way and the pH was determined until the initial pH of the diluted serum was reached. Fig. 1 shows a representative curve drawn on cross-section paper with pH on one axis and 0.2 N acid or alkali on the other. Only the curve for the back titration with alkali, curve B, would be required to calculate the content of carbon dioxide from the difference in the amount of acid and alkali added and the initial pH. It may be seen in Fig. 1 that the carbon dioxide content is equivalent to the amount of acid or alkali between curves

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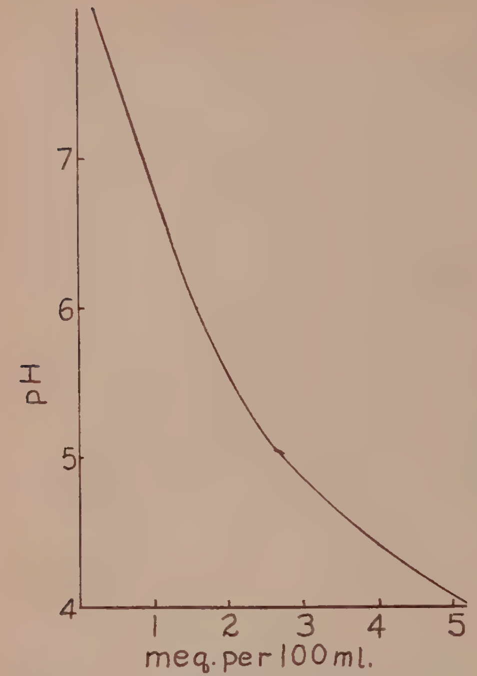

FIG. 2. Average composite curve for the back titration of plasma or serum with sodium hydroxide.

A and B at the initial pH. The concentration of acid and alkali used in the method was fixed so that the difference would always be equal to 4 milliequivalents per 100 ml of serum or plasma. If then the initial pH is assumed to be 7.60 which is the approximate average pH at room temperature, the carbon dioxide content in volumes per cent is obtained by multiplying 22.4 by 4.0 minus the difference in the amount of base in milliequivalents found on curve B between pH 7.60 and the final pH. All the B curves of samples, in which protein and carbon dioxide contents varied considerably, were corrected for the amounts of acid or alkali required to attain the same initial pH, and an average composite curve was drawn. This curve, shown in Fig. 2, in terms of milliequivalents of sodium hydroxide per 100 ml of plasma or serum, was used to calculate the subsequent values of carbon dioxide content.

We have found that a small amount of heparin sufficient to prevent clotting does not appreciably affect the initial pH or the curve, and enables a determination to be made promptly. Sodium fluoride, which inhibits glycolysis and the subsequent small change in pH, has also been found to have negligible effect. The required amount of a solution containing 100 mg of sodium heparin (Connaught) in 25 ml of 2% sodium fluoride (10 mg NaF and 0.02 mg heparin per ml of blood) was dried in tubes at 50°C, and blood drawn under oil was immediately stirred with the residues and centrifuged under oil. 0.3 ml of 0.9% sodium chloride was added to 0.2 ml samples of the plasma in small beakers. 0.1 ml of 0.1600 N hydrochloric acid was added carefully under the liquid from Kirk type micro pipettes with vigorous mixing. After thorough stirring, 0.1 ml of 0.0800 N sodium hydroxide was added in the same way. The solution was then transferred promptly to the micro MacInnis-Belcher type glass electrode, and the pH was determined. For 0.1 ml samples the procedure was the same except that 0.4 ml of sodium chloride solution and one-half the concentration of acid and alkali were used. The content of carbon dioxide was then calculated from Fig. 2 as described above.

Table I shows that the values agree quite well with those obtained by the Van Slyke manometric method and the same determinations calculated from the experimental initial pH and individual titration curves of each sample. Table II shows that the results also

TABLE I. Comparison of Values of CO$_2$ Content Obtained by the New Method with Those Found by the Van Slyke Method and Those Calculated from the Experimental Initial pH and Individual Titration Curves in 12 Experiments.

		CO$_2$ calculated from		
		Avg curve	Individual curve	
No.	pH	Vol %		Van Slyke
1	7.71	61.8	62.2	62.6
2	7.57	56.5	57.5	57.2
3	7.63	69.2	71.0	71.5
4	7.59	67.6	71.4	68.5
5	7.60	61.5	61.5	63.0
6	7.63	74.0	74.5	75.5
7	7.62	82.7	81.6	81.4
8	7.59	63.9	64.2	65.6
9	7.55	44.8	47.0	46.5
10	7.59	56.2	56.5	56.7
11	7.68	59.5	60.5	60.0
12	7.76	60.2	60.7	60.2

TABLE II. Comparison of Values of CO_2 Content Obtained by the New Method with Those Determined by the Van Slyke Method in 12 Samples of Varying Protein Content.

No.	Total protein, g/100 ml	CO_2 content, vol % New method	CO_2 content, vol % Van Slyke
1	6.5	65.7	65.6
2	6.4	63.5	63.3
3	7.2	77.6	76.7
4	6.2	65.4	65.8
5	5.4	55.7	55.5
6	5.5	57.8	56.1
7	6.7	58.9	58.4
8	5.7	70.9	72.0
9	5.1	61.3	62.2
10	5.5	61.1	61.6
11	5.2	55.5	56.1
12	7.0	60.2	62.0

agree quite well with the Van Slyke method in samples of varying protein contents. Of 307 determinations analyzed to date, 74% agreed within 2 volumes per cent of values found by the Van Slyke method. The agreement was always good between 40 and 80 volumes per cent, but there was an appreciable discrepancy in samples in which the carbon dioxide content was outside this range.

Summary. A method is described for the determination of carbon dioxide content of 0.1 and 0.2 ml samples of serum or plasma by adding 0.1 ml of standard acid and 0.1 ml of standard alkali and estimating the pH with a glass electrode. The values of carbon dioxide are then calculated from the amount of alkali found on an average composite curve for plasma or serum between an average initial pH at room temperature of 7.60 and the experimental final pH. Data are presented to show that the results obtained by this method agree quite well with values determined by the Van Slyke manometric method.

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Effect of Parathyroids on Radio-Calcium Uptake and Exchange in Rat Tissues.* (19688)

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These studies were undertaken to determine the uptake of radio calcium (Ca^{45}) by various tissues of the rat in normal and hypoparathyroid animals in the hope that further light might be thrown on the effect of the parathyroids on calcium metabolism. Until recently the Ca^{45} available for biological studies contained varying amounts of additional stable calcium. Since calcium in small amounts has marked effects on mammalian physiology, the carrier accompanying the radio-isotope could conceivably have a major influence on the results obtained. In this regard, L'Heureux *et al.*(1) found that the ratio of excreted urinary Ca^{45} to fecal Ca^{45} could be changed from 1:20 to 1:1 by increasing the amount of stable calcium administered with the radio-isotope. Lansing *et al.*(2) studied the uptake and exchange of Ca^{45} in mouse tissues in which the results obtained could be attributed to the large amount of stable calcium administered with the Ca^{45}. Tweedy *et al.*(3), in studying the excretion and distribution of radio-calcium in normal and thyroparathyroidectomized rats, administered Ca^{45} accompanied by total calcium in amounts ranging from 0.21 mg to 7.0 mg. Because of this apparent problem in the use of this isotope, it was thought best to first study the difference in the uptake by tissues of Ca^{45} when accompanied by widely varying amounts of carrier calcium.

Material and methods. A total of over 150 male rats, weighing between 200 and 250 g,

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* Supported by a grant from the U. S. Atomic Energy Commission.

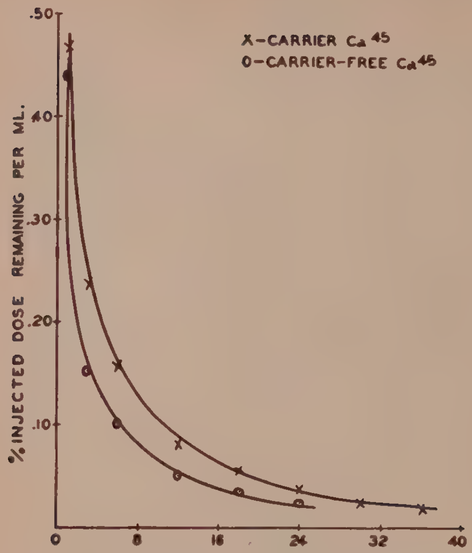

FIG. 1. Disappearance of Ca^{45} from plasma of normal rats after administration of 20 μc carrier and carrier-free Ca^{45}.

were used, and each point on the graphs included represents an average of from 4 to 10 animals. The Ca^{45} was obtained from the Atomic Energy Commission and administered intraperitoneally as $CaCl_2$. Most of the animals were given the radio-isotope accompanied by approximately 0.5 mg of total calcium. Since preliminary work showed the uptake to be similar to that of carrier-free Ca^{45}, this is considered *physiologically* carrier-free; and this term, though not technically correct, is used for the sake of convenience in the remainder of this paper. For comparison, the Ca^{45} was also administered accompanied by 10 mg of stable calcium. The term, carrier-Ca^{45}, will be used in this regard. In all cases, the total radio-activity per animal amounted to approximately 20 μc. Dilutions of the injection solution were used as standards for measurement of radio-activity, and all results are given in percentage of the injected dose. The radio-activity measurements were done by standard procedures and corrected for self-absorption. Total serum calciums were determined by the method of Clark and Collip (4). The parathyroids were removed individually following the procedure described by Greep (5). Since some accessory parathyroid tissue is usually present in the rat, the criterion used for determining hypoparathyroidism was the drop in serum calcium to 7 mg % or lower. As a standard procedure, the animals were allowed 3 days after the operation for recovery and then starved overnight before use in the experiments. The control animals were also starved a similar period. Radioactivity determinations were done on both the tibia and the femur, and radio-autographs of the latter were made after trimming the bone on a freezing microtome to obtain longitudinal sections.

Results. The data summarized in Fig. 1, 2, and 3 show a comparison of the uptake of Ca^{45} administered under the two conditions discussed above. Fig. 1 shows the disappearance of radio-activity from the blood. As might be expected the carrier-Ca^{45} disappeared from the blood somewhat slower than did the carrier-free Ca^{45}. Total serum calciums, which were run concurrently, remained normal in the carrier-free group. However, in the carrier group, these values rose to above 15 mg % within the first hour after injection but returned to normal within 6 hours. It is of interest to note that these changes in serum

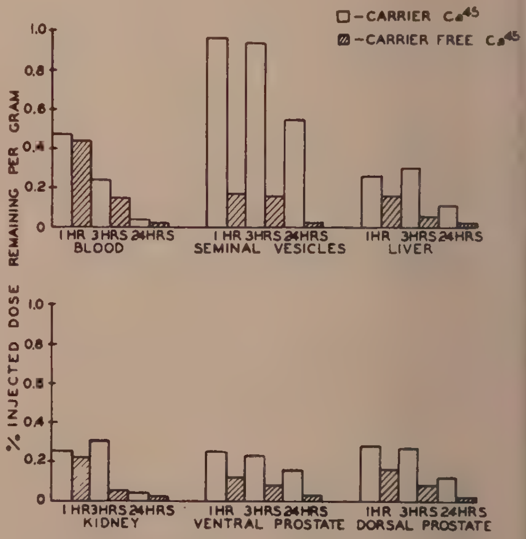

FIG. 2. Distribution of Ca^{45} in tissues of normal rats after administration of 20 μc carrier and carrier-free Ca^{45}.

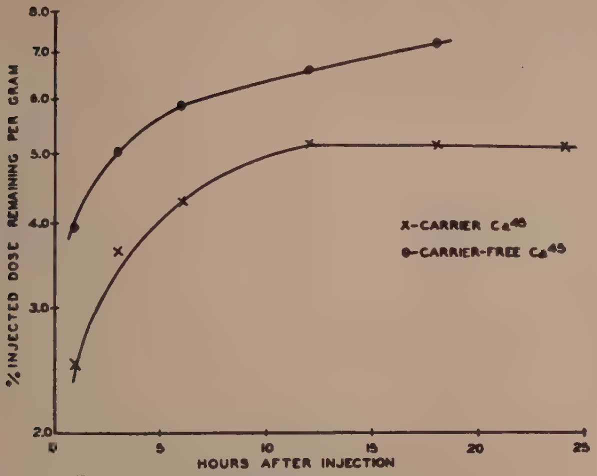

FIG. 3. Uptake of Ca^{45} in bone of normal rats after administration of 20 μc of carrier and carrier-free Ca^{45}.

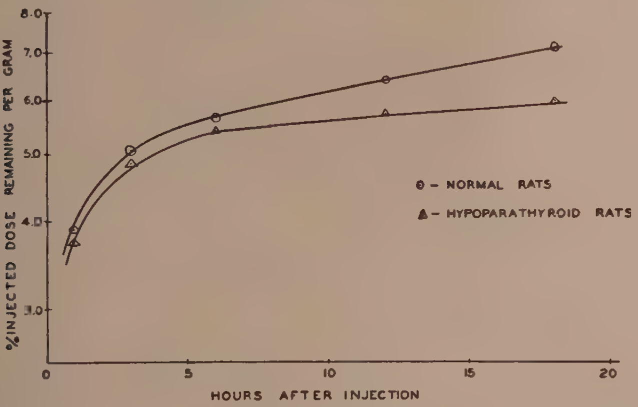

FIG. 4. Uptake of Ca^{45} in bone of normal and hypoparathyroid rats after administration of 20 μc carrier-free Ca^{45}.

calcium were not reflected in the radio-activity disappearance curves.

In Fig. 2 is given the Ca^{45} distribution for a few selected tissues. It can be seen that with the carrier-free Ca^{45} the tissue activity approximates but never exceeds the radio-activity of the blood at any given time. The uptake by the tissues of these animals appears to be a function of the blood supply and indicates very little actual calcium deposition. When carrier-Ca^{45} is administered, the situation is strikingly different. Twenty-four hours after injection, the activity of all the tissues studied surpassed that of blood, indicating deposition of the administered calcium. This was emphasized in the seminal vesicles in which the uptake approached one-fourth of that seen in long bone. The significance of this unusual uptake by the seminal vesicles is under study.

The uptake of radio-calcium by bone (Fig. 3) in the carrier-group is significantly less than that in the carrier-free group. However, due to the low specific activity of the carrier-Ca^{45} and the increase in the serum calcium in those animals receiving it, it is apparent that despite the lower bone activity, considerably more total calcium is taken up by the bones

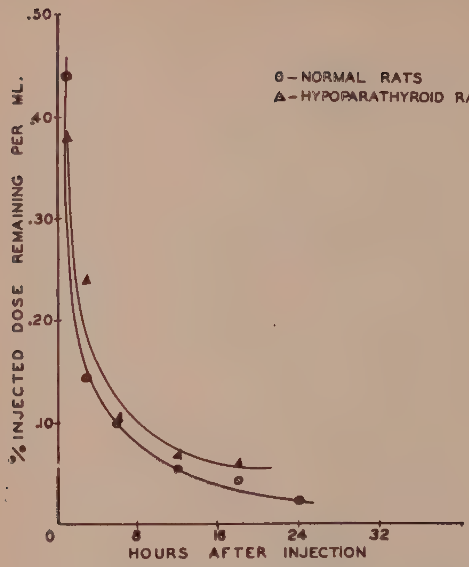

FIG. 5. Disappearance of Ca^{45} from plasma of normal and hypoparathyroid rats after administration of 20 μc carrier-free Ca^{45}.

of the carrier-Ca^{45} group.

The uptake of carrier-free Ca^{45} by bone can be divided into two time periods. The first 6 hours can be considered the "exchange period" in which the radio-calcium is equilibrating with calcium in blood, soft tissues and that portion of the calcium of bone which is either in ionic form or in loose organic union. The straight line portion of the graph following this period is termed the "turnover rate" portion and is considered to indicate the rate at which calcium is being actively laid down by the long bone.

For our comparison of normal and hypoparathyroid rats, only the carrier-free Ca^{45} has been used. Fig. 4, 5, and 6 summarize this work. In Fig. 4, the uptake by long bone is illustrated. It will be noted that while there is little or no difference during the "exchange" phase in the uptake of radio-calcium, the straight-line portion of the graph, indicating turnover rate, in the hypoparathyroid condition markedly falls away from the controls. This lower turnover rate in the bones of the experimental animal is substantiated by the slightly slower rate of disappearance of the radio-calcium from the blood (Fig. 5) and the corresponding higher uptake in those tissues studied (Fig. 6). All radio-autographs made of bones showed a similar deposition of Ca^{45} in the periosteum and the epiphyseal plates, indicating that the differences in uptake noted were in degree only and that metabolism of the bone may not have been affected.

Discussion. The use of the radio-isotope of calcium, Ca^{45}, presents several problems. Due to the effect of calcium on the cardiovascular system, very little carrier is required to markedly alter at least some of the physiological processes of the animal. The work presented here shows the differences in uptake of radio-calcium which can be produced by using two extreme conditions: one, the administration of an amount of calcium sufficient to raise the blood calcium level of the animal; the other, Ca^{45} accompanied by the minimum amount of carrier present in the Oak Ridge material available for our use. Not only could it be shown that the carrier present with the radio-isotope drastically affected the distribution of Ca^{45}, and that the carrier masked some of the uptake indicated by the radio-isotope, but also that changes

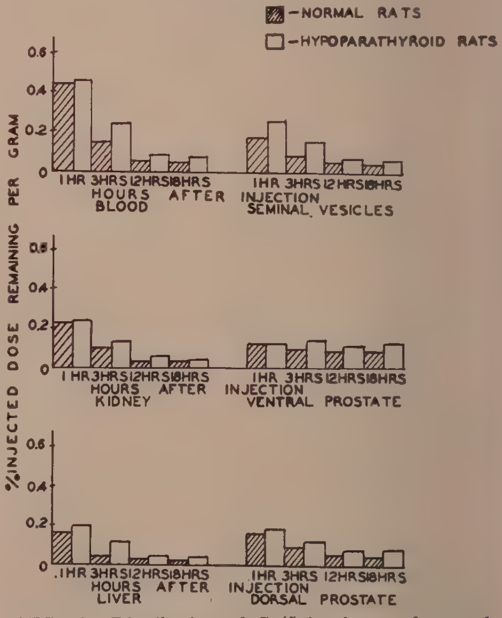

FIG. 6. Distribution of Ca^{45} in tissues of normal and hypoparathyroid rats after administration of 20 μc carrier-free Ca^{45}.

produced by the carrier were not necessarily portrayed by the radio-isotope. These are problems in common with any radio-isotope which is being used in biological experiments, but particularly important with one as physiologically sensitive as calcium. However, though the uptake of carrier-Ca^{45} as presented here is not a physiological situation due to the excessive amount of calcium given, the use of carrier-Ca^{45} may prove to be a useful tool in determining the ability of bone to handle large amounts of calcium.

These preliminary experiments with normal and hypoparathyroid rats did not yield any information having a direct bearing on the physiological functioning of the parathyroid hormone. However, they do illustrate very clearly the difference in bone uptake and turnover of calcium in the two situations. All radio-autographs showed the primary sites of calcium entry into the bone to be at the epiphyses and in the periosteum and indicate that this process is affected only in degree in the various situations tested by these experiments. Whether or not one could conclude from this that the basis of the action of the parathyroids is other than in bone itself is doubtful, but it is hoped that further experiments now in progress will bear on this point.

Summary. This study has presented two factors which can markedly affect the uptake and distribution of Ca^{45} in tissues of the rat. The first of these was concerned with the influence of the carrier calcium accompanying the radio element and emphasizes the need for carefully controlled experiments if even small amounts of carrier are administered with the Ca^{45}. The second factor was concerned with the influence of parathyroidectomy on the uptake of Ca^{45}. The uptake by bone was divided into two time periods, the "exchange period" and the "turnover rate" portion. The exchange period was not markedly affected by the absence of the parathyroids while the turnover of calcium in the bone was at a significantly lower rate.

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Effect of Sympathomimetic Amines upon Amino Acid Nitrogen Content of the Blood. (19689)

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The lowering of blood amino acid nitrogen which follows upon the administration of insulin to man and experimental animals (1-8,11) is also observed when epinephrine is injected(9-11). The insulin effect persists even when the hypoglycemia is prevented by the oral administration of glucose(12) or by the simultaneous administration of epinephrine(9). Insulin hypoaminoacidemia is not observed, however, in the adrenodemedullated rabbit(13) although, in such animals, epinephrine continues to evoke its characteristic lowering of the concentration of blood amino acids(13). It was, therefore, concluded by Luck and associates(10,11,13), that insulin hypoaminoacidemia is, in reality, the result of stimulation of the adrenal medulla, and that the epinephrine thus secreted evokes the observed response or stimulates another gland of internal secretion in a chain of hormonal interactions.

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TABLE I. Sympathomimetic Amines and Hypoaminoacidemia. Blood amino nitrogen in percentage of initial value.*

Substance administered	Dose, mg/kg	Hr after administration					
		0	½	¾	1	2	4
Epinephrine HCl	.2	100	91.8 (3.7)		80.1 (6.1)	71.2 (3.4)	67 (4.7)
Ephedrine H_2SO_4	2	100	100.9 (4.8)		95 (3)	94.5 (10)	101 (5.5)
dl-Desoxyephedrine†	20	100		100.5		99.1	98.3
dl-Arterenol HCl	2	100		99.3 (1.1)		99.1 (.7)	96.6 (.2)
Phenylethylamine HCl	20	100		102.7 (4)		106.2 (6.5)	110.3 (5.6)
Propadrine HCl‡	5 15	100		102.4 (1.4)		102.4 (1.9)	101.7 (.7)
Neosynephrine HCl	10	100		99.9 (3)		100.2 (4.9)	103.8 (2.2)
Tuamine H_2SO_4	10	100		99.8 (3)		101.4 (.8)	102.5 (3.8)

* Each value is the avg of determinations on 4 rabbits, the figures in parentheses representing the avg deviation from the mean.

† One animal only.

‡ Two animals received the lower dose, 2 the higher.

The important role of epinephrine in the induction of hypoaminoacidemia led us to inquire into the possible effect of various sympathomimetic amines, as substitutes for epinephrine, in producing an amino acid lowering.

Materials and methods. Rabbits were used as the subjects in this series of experiments. A preliminary blood sample was obtained from the ear, and the compound to be tested was administered subcutaneously. The dosage given for the various drugs was roughly equivalent to that given for epinephrine in terms of pressor activity. Blood samples were then taken from the ear at the time intervals noted below. The amino acid nitrogen content of these blood samples was determined colorimetrically using the Folin method as modified by Danielson(14).

Conclusions. From the data obtained it is seen that only epinephrine displays the ability to lower the blood amino acid nitrogen. This confirms the observations of Luck and associates(9-11). While 3 out of 4 rabbits displayed a moderate increase in amino acid N following phenylethylamine administration, the significance of this is not apparent. The fluctuations obtained with the other sympathomimetic drugs are not great enough to exclude experimental error.

We are greatly indebted to Dr. M. L. Tainter and the Sterling-Winthrop Research Institute for donating most of the amines used in this investigation.

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Nucleic Acid Changes in Sarcoma 180 Following Administration of Aminopterin.* (19690)

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It has been noted that aminopterin (4-amino-pteroylglutamic acid) retarded the growth of transplantable sarcoma 180 in mice and altered the histology of the tumor(1). The histologic changes observed in treated tumors included a marked decrease in the number of mitoses, the appearance of many cells with very large altered nuclei, and either diminution or vacuolization of the cytoplasm. As a result of these histologic observations, chemical and histochemical studies were initiated to determine whether demonstrable changes in the amounts of pentose- and desoxypentose nucleic acids (PNA and DNA) had occurred after administration of the folic acid antagonist.

It was recognized that chemical analysis of homogenized tumor tissue could only furnish evidence of gross changes induced by chemotherapy. Concomitant histochemical studies were carried out to measure alterations occurring in individual nuclei(2). Tissue homogenates of tumors usually contain a mixture of several types of cells. The sarcoma 180 was harvested in the pre-necrotic stage (within 10 days after implantation) and the cellular composition was predominantly tumor cells. It is believed that the changes observed in the PNA and DNA contents are referable to the tumor cells.

The general procedures employed, such as the care of the mice, implantation of tumor and administration of aminopterin, were similar to those previously described(1). At the termination of each experiment the mice were sacrificed by cervical dislocation, the tumors were dissected free from adhering tissue and blotted with filter paper to remove tissue fluids. The tumors were placed in tightly stoppered flasks immersed in a CO_2 ice-acetone bath, and stored in a CO_2 ice cabinet until analyzed.

The tissues were analyzed for both types of nucleic acid according to the method of Schneider(3). Nitrogen was determined by the micro-Kjeldahl method. A model DU Beckman spectrophotometer, employing one cm cuvettes, was used for all the colorimetric measurements.

Results. The results of 4 separate chemotherapeutic experiments are presented in Table I. There are 15 individual analyses of control and 15 of aminopterin-treated tumors. The number of replicate determinations within each experiment was determined by the number and size of the tumors available. It has often been noted that the growth of sarcoma 180 is subject to variation which could be attributed to such factors as host response and the size of the initial tumor inoculum. Because of this biological variability the treated tumors of each experiment were compared with concomitant controls. Another variable factor considered was the range of nucleic acid values for individual samples found by Schneider(3). Therefore, each analysis was performed on a pool of tumor tissue consisting of 10 to 40 tumor samples obtained from control or treated animals. Thus, within each experiment the control and treated material represented a fair sampling of the population of that particular trial.

One may employ the nitrogen content per gram of tissue as a rough index of the cellularity and degree of hydration of the tumors.

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TABLE I. Summary of Nucleic Acid Determinations on Sarcoma 180.

		Saline control					Aminopterin treated					Mean % change from control value	
Exp. (1)	Age of tumors days (2)	Sample No. (3)	N, g* (4)	PNA, mg (5)	DNA, mg* (6)	PNA / DNA (7)	Sample No. (8)	N, g* (9)	PNA, mg* (10)	DNA, mg (11)	DNA / PNA (12)	DNA (13)	PNA (14)
EA 181	6	1	1.81	522	473	1.10	2	2.40	482	368	1.31	–24.6	–10.2
		1A	"	533	499	1.11							
	10	3	2.20	608	548	1.11	4	2.12	597	497	1.20	– 6.5	– 7.4
		3A	"	684	563	1.22	4A	"	626	536	1.17		
		3B	"	688	544	1.27							
	10	5	2.22	681	592	1.15	6	2.06	709	503	1.41	– 7.4	+ 3.6
		5A	"	701	512	1.37	6A	"	724	518	1.40		
EA	9	7	1.90	655	546	1.20	8	1.81	648	473	1.37	–14.3	– 4.1
		7A	"	655	560	1.17	8A	"	608	465	1.28		
WC	10	9	2.52	962	655	1.47	10 †	2.38	919	553	1.66	–14.3	– 6.7
		9A	"	990	646	1.53	10A†	"	903	563	1.60		
	10	"	"	"	"	"	11 ‡	2.43	1009	624	1.76	– 6.9	+11.3
							11A‡	"	1072	588	1.82		
BG	10	12§	2.30	762	609	1.25	13 §	2.18	854	617	1.38	0	+14
				730	603	1.21			846	592	1.43		
	10	14‖	2.46	722	624	1.24	15 ‖	2.46	845	601	1.41	– 4.3	+11.1
				753	616	1.22			851	584	1.46		
Mean ± S.D.						1.24 ± .13					1.44 ± .21		

* Per 100 g tissue (wet wt). † Selected large tumors (see text). ‡ Selected medium tumors (see text). § Stock tumors implanted (see text). ‖ 6th generation of aminopterin-treated tumors implanted.

The ungrouped nitrogen values (col. 4 and 9) for all the observations are distributed about a mean of 2.20 g N per 100 g tissue (wet weight) with a standard deviation of 0.23. If the values are separated into control and treated groups, the means are 2.18 and 2.23 respectively. These values are not significantly different from each other and are within the range observed with liver or kidney tissue. From the individual analyses presented in Table I it may be noted that there is no consistent change in the nitrogen content of the treated tumors as compared to the controls. The total nucleic acid content (PNA and DNA) per 100 g of tumor tissue is approximately 1.2 g, equivalent to 0.2 g nitrogen. The detection of significant deviation in the nucleic acid content by means of nitrogen determinations is impossible because the standard deviation (0.23) for the total nitrogen content of tumor tissue noted with the ungrouped data is equal in magnitude to the total nucleic acid nitrogen.

The nucleic acid determinations and the alterations observed after antagonist administration are summarized in Table I. The observations may best be discussed under each experiment.

EA 181. Two hundred female mice were implanted bilaterally with tumor. One day after implantation and on 4 consecutive days, 120 mice received subcutaneous injections of aminopterin in a dosage of 0.3 mg per kg daily. The remaining 80 mice served as controls and received only injections of normal saline solution. On the sixth day after implantation, approximately one-half of each group was sacrificed. The remaining mice in the experimental group received 0.2 mg per kg of drug on the sixth and eighth days, while the controls received saline. All these mice were sacrificed on the tenth day after implantation. The material obtained from treated mice bearing 6-day-old tumors was sufficient for only a single nucleic acid determination (Table I, Col. 8, Sample 2). It can be seen that the content of both PNA and DNA (Col. 10 and 11) in the tumors decreased compared to the 6-day control tumors (Col. 5 and 6, Sample 1). The decrease in DNA was greater than that of PNA (Col. 13 and 14). The 10-day tumors were analyzed on 2 separate occasions. The first set of analyses (Samples 3 and 4) showed a diminution of both PNA and DNA among the treated cells (Col. 13 and 14). A comparable decrease of both PNA and DNA in the tumors after aminopterin administration was only observed in this analysis and was not encountered again in any other experiments. The second analysis (Samples 5 and 6) showed a decrease in the DNA content of the treated tumors equal to that observed in the previous analysis but the PNA content was somewhat increased (Col. 13 and 14).

EA 191. The animals in this experiment received 5 daily injections of 0.3 mg of aminopterin per kg and were sacrificed on the ninth day after tumor implantation. The PNA and DNA in the tumors were both reduced after treatment, but the reduction was disproportionately great for DNA (Col. 13 and 14).

WC III. It had been repeatedly observed that treated tumors varied appreciably in size and therefore tumors were grouped for analysis at autopsy into 3 categories: large, medium and small. Control tumors in all experiments fell into the "large" class and these were not separated at this time. The tumors classified in the "small" group did not provide enough material for chemical analysis.

The results (Table I, Samples 9, 10, 11) show that in both the large and medium groups the DNA content decreased from that in the controls (Col. 13 and 14). The PNA content was either somewhat diminished or increased when compared to the controls (Col. 14). The ratio of PNA to DNA content of the grouped tumors varied in the following order: "medium" > "large" > control (Col. 12 and 7). These results are consistent with the observation that those tumors which were most affected by aminopterin would be smallest in size. The smaller tumors from treated mice appear to show the largest decrease in DNA relative to PNA.

BG. It is well known that bacteria can develop or acquire resistance to chemotherapeutic agents(4). Burchenal *et al.* have also shown that a mouse leukemia could develop resistance to the folic acid antagonist, amethopterin(5). It seemed worthwhile to see whether sarcoma 180 would show changes in its properties upon serial passage through mice treated with aminopterin. The tumor was transplanted every 10 days for 6 transfers in "A" strain mice. They received 0.5 mg of aminopterin per kg body weight daily for a 6-day period. Tumors which had been exposed through 6 passages in treated mice were then implanted into CF1 mice. One group received aminopterin treatment (Sample 15), the other received saline injections (Sample 14). Tumors which had not been exposed to aminopterin therapy were also implanted into CF1 mice and these were similarly divided into a treated (Sample 13) and control (Sample 12) group.

From the results presented in the table, it can be seen that previous exposure to aminopterin had no effect on the tumor response to further treatment. In both groups the PNA:DNA ratio of the treated tumors increased in the same manner over the controls (Col. 12 and 7). These experiments and additional transfers in the presence of aminopterin did not indicate that tolerance to the antagonist developed when tumor size and weight were measured.

Discussion. An examination of the PNA:DNA ratios of all the experiments indicates that a consistent difference exists between treated and control tumors. The means and standard deviations of these ratios are presented in Table I. The coefficient of variation for the control groups is 10% while for the aminopterin-treated groups it is 15%. These coefficients are not very large considering the factors of variability previously mentioned. The difference of the means, 0.20, is more than 3 times 0.065, the standard error of the difference(6).

The deviations from the control values may be examined to note the factors responsible for the change in the ratio of PNA to DNA (Col. 13 and 14). Following aminopterin therapy the reduction observed in DNA varied from 0 to 25%. The PNA content of treated tumors was lower than that of the controls in 4 instances (–4 to –10%) and higher in 4 (+3.6 to 14%). With the possible exception of analysis No. 4, DNA was reduced disproportionately more than PNA in each of the experiments. Since the DNA analyses in Samples 4 and 6 agree (Exp. EA 181, Col. 11) there is a possibility that the PNA analysis in Samples 3 or 4 (Exp. EA 181, Col. 5 and 10) was in error. From these data it appears that aminopterin administration interferes with the synthesis of desoxypentose nucleic acid in the tumor cells. The synthesis of pentose nucleic acid was not affected to the same degree as DNA and in several experiments no inhibition of PNA synthesis was noted. These results supplement the findings of Prusoff, Teply and King, who observed that *L. casei* grown on folic acid-deficient media had a lowered DNA but unaltered PNA content(7). Histochemical studies are in progress in which the relative nuclear volume and the DNA content of the individual nuclei are being measured. It is hoped that these latter studies will more clearly define the mechanisms within the cell responsible for the reduced DNA content in aminopterin-treated tumors.

Summary. The pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA) content of sarcoma 180 tumors has been determined in mice receiving aminopterin and in controls. The chief alteration associated with aminopterin administration was an increase in the ratio of PNA to DNA due to a reduction in the relative DNA content. Tumors which had been serially transplanted into aminopterin-treated mice, when used as inocula, showed no difference when compared to stock tumors in their subsequent response to aminopterin.

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A Method for the Biological Preparation of Deuterium-Labeled Cholesterol From the Hen's Egg.*† (19691)

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It is well known that both deuterium-labeled acetate and deuterium oxide can be used by animals in the synthesis of cholesterol in tissues(1,2). Investigators have shown that the deuterium is present both in the ring and in the side chain(1) and that the incorporation of deuterium into the cholesterol is not simply an exchange reaction(2).

In work reported by Kritchevsky *et al.* in which sodium-acetate 1-C^{14} was fed to laying hens, it was found that there was a rapid incorporation of the acetate into various fractions of the egg with the specific activity higher in the cholesterol than in any other fraction(3,4). In this laboratory, experiments

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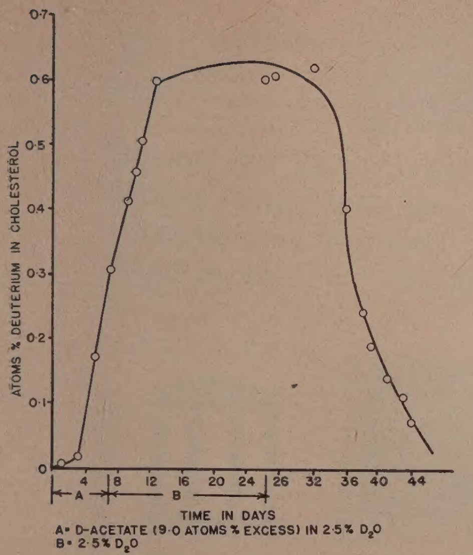

INCORPORATION OF DEUTERIUM INTO CHOLESTEROL OF YOLK OF HENS EGG

FIG. 1

have been conducted to prepare deuterium-labeled cholesterol by a similar treatment.

Experimental procedure. Two laying hens were given an isotonic solution of sodium-acetate, labeled with deuterium (9.0 atoms % excess) in the methyl position, dissolved in 2.5% deuterium oxide in their drinking water for 7 days. Subsequently, only the 2.5% deuterium oxide was given for 19 days. Eggs were collected as available starting from the first day for a period of 44 days. To separate the yolk from the white, the eggs were immersed in boiling water for 10 minutes after which the coagulated white portions were discarded. Cholesterol was extracted from the yolks by a Soxhlet extraction for 8 hours using 3:2 alcohol-ether solution. On analysis of 34 eggs(5) the individual yolks were found to contain from 200-250 mg cholesterol. Cholesterol was precipitated as the digitonide, and analyses for deuterium were done as previously reported by this laboratory(6).

Results and discussion. Fig. 1 shows the distribution of deuterium in the cholesterol of the eggs over the experimental period. As has previously been observed in the carbon-14 labeled acetate studies of Kritchevsky(3), as early as the first day after the administration of the deuterium labeled acetate and deuterium oxide, deuterium appeared in the cholesterol of the yolk, substantiating the very rapid incorporation of these substances. Two sources of deuterium were used to increase the amount of incorporation of the isotope since it has been shown that the deuterium content of the cholesterol is much smaller if only deuterium oxide is administered(7). It can be seen that the maximum incorporation of deuterium continued from the 12th to the 32nd day, in spite of the fact that the source of deuterium was discontinued after the 26th day, after which the amount of label decreased rapidly.

Summary. A simple, relatively inexpensive method for the biological preparation of deuterium-labeled cholesterol from hen's eggs has been described. The daily incorporation and subsequent disappearance of the deuterium into the cholesterol has been followed.

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